# Analysis of the mammary gland specific effect of endothelin-1 in transgenic mice

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*Dedicated to the memory of my dear father*

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# Zusammenfassung

Endothelin-1 (ET-1) ist ein gefäβaktives Peptid, welches zusätzlich verschiedenste nicht kardiovaskuläre physiologische und pathophysiologische Effekte besitzt. So wurde z.B. beschrieben, dass ET-1 in der Brustdrüse während der Schwangerschaft und Stillzeit exprimiert wird. Zusätzlich zu den bekannten Nährstoffen und Wachstumsfaktoren konnte auch ET-1 in der Muttermilch nachgewiesen werden, was auf eine physiologische Rolle von ET-1 für die Laktation und den säugenden Nachwuchs hinweist. In der vorliegenden Arbeit sollte die Funktion von ET-1 in der Brustdrüsenentwicklung mit Hilfe von ET-1 transgenen Mäusen aufgeklärt werden. Die eingesetzten transgenen Tiere überexprimieren humanes ET-1 mit den entsprechenden 5'- und 3' regulatorischen Sequenzen. Mit Hilfe dieser Strategie sollen die ET-1 spezifischen Funktionen während der Brustdrüsenentwicklung untersucht werden.

Transgenes ET-1 wurde während der Tragzeit und Stillzeit in der Brustdrüse detektiert. Die Ergbnisse zeigten, dass säugende Neugeborene der ET-1 transgenen Mäuse eine geringere Gewichtszunahme und eine erhöhte Mortalität aufwiesen, welches auf einen Laktationsdefekt hinweist. Die histologische Untersuchung der Brustdrüse während der Tragzeit ergab eine reduzierte Milchkanalausbildung, kollabierte und nicht expandierende Alveoli, vermehrte Adipozytenausbildung und fortbestehende zytoplasmatische Lipidtropfen (CLDs). Zusätzlich war die Expression des Milchproteins WAP reprimiert. Interessanterweise wurde diese Repression nicht durch STAT5, einem beschriebenem Regulator der Milchproteinexpression und Alveolarexpansion, vermittelt, da dessen Aktivität unverändert war. Als Konsequenz dieses Laktationsdefekts konnte eine verfrühte Rückbildung der Brustdrüse festgestellt werden. Diese ging mit einer erhöhten Expression von STAT3 einher. Interessanterweise wies der bekannte Aktivator von STAT3, LIF, ebenfalls eine gesteigerte Aktivität auf, sowohl während der Tragzeit als auch während der Laktation.

Zusätzlich zu den beschriebenen Defekten bei der Milchabgabe zeigten histologische Untersuchungen der Brustdrüse eine Laktationshyperplasie während der mittleren Laktationsphase. In diesem Zusammenhang wird darauf hingewiesen, dass ET-1 Rezeptoren, neben den klassischen Signalwegen dieser G Protein-gekoppelte Rezeptoren, auch mit Tyosinkinaserezeptoren wie z. B. dem EGFR interagieren können. Brustdrüsen von ET-1 transgenen Tieren zeigten eine erhöhte Aktivität sowohl von EGFR als auch von ERK1/2, welches im Zusammenhang mit dem hyperplastischen Phänotyp stehen könnte. Die mögliche tumorfördernde Wirkung von ET-1 wird ferner durch die erhöhte Expression von Amphiregulin, einem EGFR-Liganden, während der Tragzeit und der Laktation verstärkt.

Zusammenfassend konnte festgestellt werden, dass ET-1 sowohl die Milchsekretion als auch den Milcheinschuss negativ beeinflusst, so dass eine ausreichende Versorgung säugender

Jungtiere in der 1. Hälfte der Laktationsperiode nicht mehr gewährleistet ist. Zusätzlich verursachte ET-1 eine Laktationshyperplasie, welche auf die Induktion der EGFR-Achse zurückzuführen ist. Zusammenfassend kann somit festgestellt werden, dass die Ergebnisse auf eine wichtige Rolle von ET-1 in der Brüstphysiologie des Säugers hinweisen.

Schlagwörter:

Endothelin 1, Milchdrüse, Laktationsdefekt, Laktationshyperplasie, Transaktivierung der EGFR

# Summary

Endothelin-1 (ET-1) is a potent vasoactive peptide having wide physiological effects on vascular homeostasis and on a variety of pathophysiological processes unrelated to cardiovascular system. It has been noted that ET-1 is expressed in mammary glands during pregnancy and lactation periods. Furthermore, ET-1 is secreted into milk, suggesting additional physiological roles in the lactating mother and in the suckling neonate. Hence, the present study was proposed to elucidate the possible functional roles of ET-1 in mammary gland development employing ET-1 transgenic mice. ET-1 transgenic mice had been generated by using a human genomic ET-1 construct containing 5´ and 3´ regulatory sequences. This transgenic construction strategy grants to analyse the specific functions of ET-1 in normal mammary gland physiology.

The transgene expression was found in mammary gland during pregnancy and lactation. ET-1 transgenic mice exhibited a lactational incompetence with reduced weight gain and increased mortality of their newborns, as a result of a secretory defect. In virtue of this defect, ET-1 transgenic mammary glands histologically revealed a reduced ductal outgrowth, collapsed alveoli with a reduced expansion capacity, increased adipocyte accumulation, and persistence of cystoplasmic lipid droplets (CLDs) during lactation. In addition, the expression of the milk protein, WAP, was found to be constantly suppressed in ET-1 mammary glands although the activity of STAT5, which is known to be a regulator of the expression of milk proteins and alveolar expansion, was found to be normal. Furthermore, as a consequence of the secretory defect, ET-1 transgenic mammary glands exhibited focal precocious involution during early stages of lactation along with an increased activity of STAT3. Consistently, the known activator of STAT3, LIF, was strongly upregulated during lactation and pregnancy.

Besides the secretory defect of ET-1 transgenic mammary glands, histological analysis revealed a local lactational hyperplasia during the middle of lactation. Alternatively to the classical G protein-coupled receptors GPCR signalling pathways, endothelin receptors are able to communicate with tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR) for which the term receptor transactivation was coined. Mammary glands of ET-1 transgenic animals exhibited an increased activity of the EGFR and ERK1/2, which could contribute to the observed hyperplastic phenotype. In support of the potential tumourigenicity of ET-1, one of the EGFR ligands, amphiregulin, was found significantly upregulated in ET-1 transgenic mammary glands, both during pregnancy and lactation periods.

In summary, high levels of ET-1 affect the secretion and the milk let down process. Consequently the normal support of milk for the suckling neonates is severely impaired during the first half of the lactation period. In addition, ET-1 caused lactational hyperplasia in the mam-

mary glands due to the induction of the EGFR axis. This suggests an important role for ET-1 in mammary gland physiology.

Keywords:

Endothelin 1, Mammary gland, Lactational defect, Lactational hyperplasia, EGFR transactivation

# Table of Contents

# Abbreviations

| | |
|---|---|
| APS | Ammonum peroxodisulfat |
| ATP | Adenosin triphosphate |
| BMI | 4-(3-butoxy-4-methoxy-benzyl) imidazolidone |
| BrDU | 5-bromo-2'-deoxyuridine |
| BSA | Bovine Serum Albumin |
| cAMP | Cyclic Adenosin mono phosphate |
| DEPC | Dimethyl pyrocarbonate |
| DMEM | Dulbelco Modified Eagle Medium |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxy nucleotide tri phosphate |
| DTT | Dithiothreitol |
| ECL | Enhanced chemiluminescence |
| EDTA | Etilen diamin tetra acetic acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| F primer | Forward primer |
| FCS | Foetal Calf Serum |
| h | hour |
| $H_2O_2$ | Hydrogen Peroxyde |
| HCl | Hydrocloride |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HRP | horseradish peroxidase |
| IBMX | Isobutylmethylxanthine |
| Kb | kilo base |
| kDa | kilo Dalton |
| $LN_2$ | Liquid nitrogen |
| M | Molarity |
| mA | milliamper |
| mg | milligram |
| $MgCl_2$ | Magnesium chloride |
| ml | millilitre |
| µg | microgram |
| µM | micromolar |
| min | minute |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NaCl | Sodium chloride |

| | |
|---|---|
| NaF | Sodium floride |
| $NaN_3$ | Sodium azide |
| NaOH | Sodium hydroxide |
| $NA_3VO_4$ | Sodium ortovanadate |
| nm | nanometer |
| PAGE | Poly Acrilamid Gel Electrophoresis |
| PBS | Phosphate buffered Saline |
| PCR | Polymerase Chain Reaction |
| pH | Preponderance of hydrogen ions |
| pmol | picomol |
| PVDF | Polyvinylidene Fluoride |
| R primer | Reverse primer |
| RIN | RNA integration number |
| RIPA | Radio Immuno Preciptation Assay |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| SDS | Sodium Dodesil Sulphat |
| TBS | Tris buffered Saline |
| TEMED | Tetramethylethylenediamine |
| Tris | tri-(hydroxymethyl)-aminomethane |
| TM | Temparature Melting |
| UV | Ultra violet |
| V | Voltage |
| v/v | volume/volume |
| w/v | weight/volume |

**Symbols of nucleic acids**

| | |
|---|---|
| *A* | *Adenosine* |
| *C* | *Cytidine* |
| *G* | *Guanosine* |
| *T* | *Thymidine* |

# 1 Introduction

## 1.1 The mouse mammary gland

The main characteristic of the mammary gland is the provision of nourishment to offspring with its special ability of production and secretion of milk. Most of its functional and structural development undergoes a complex differentiation and systemic hormonal regulation in the adult organism.

Two structural components, epithelial parenchyma and its surrounding adipose stroma, concretize the mammary gland. The parenchyma refers to the system of branching ducts and alveoli that are the sites of milk secretion. The stroma provides substrates for maintaining of parenchyma and an environment where parenchyma can grow and function. The mammary glands structural and functional development can be categorized into six stages: embryonic, postnatal, puberty, pregnancy, lactation and involution (Figure1).

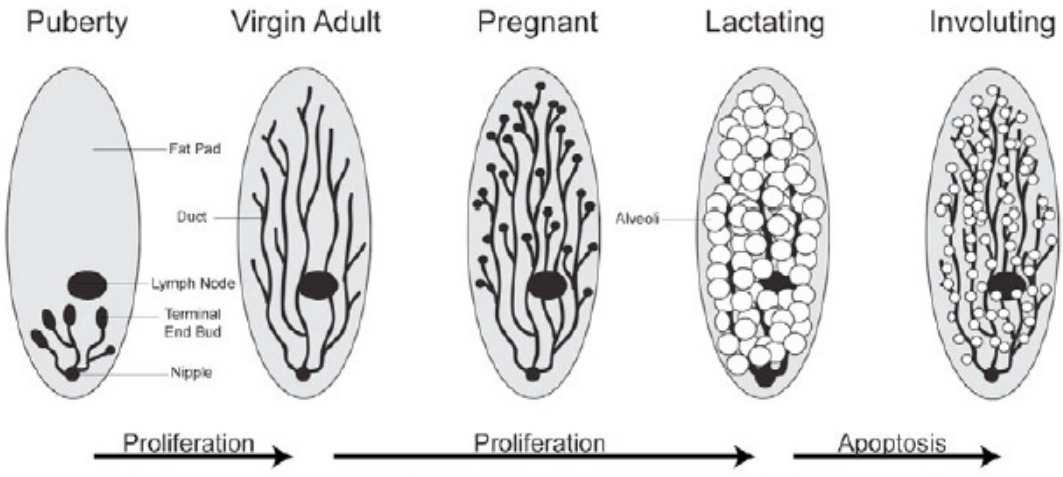


*Figure 1: Schematic illustration of the development of the mouse mammary gland. Each stage of the scheme is accompanied by timepoints of mammary gland development (Taken from (Andrechek, et al., 2008).*

### 1.1.1 Embryonic mammary gland development

Around embryonic day 10, the first sign of embryonic mammary development begins with an epidermal ridge (Figure 2, orange) that develops slight thickening and stratification of the ectoderm on the ventral surface of the embryo between the anterior and posterior limb buds (Figure 2, grey).

Ectodermal cells within this ridge disassemble into individual placodes (orange) and invigilate into the underlying mammary mesenchyme (Figure 2, blue) to form mammary buds. On em-

bryonic day 15.5, the mammary epithelium (orange) elongates into the mammary fat pad precursor (green) and begins to form a rudimentary ductal system. Overlying epidermis of the mammary mesenchyme is transformed into the nipple and areola (Figure 2, purple) on embryonic day 18.5.

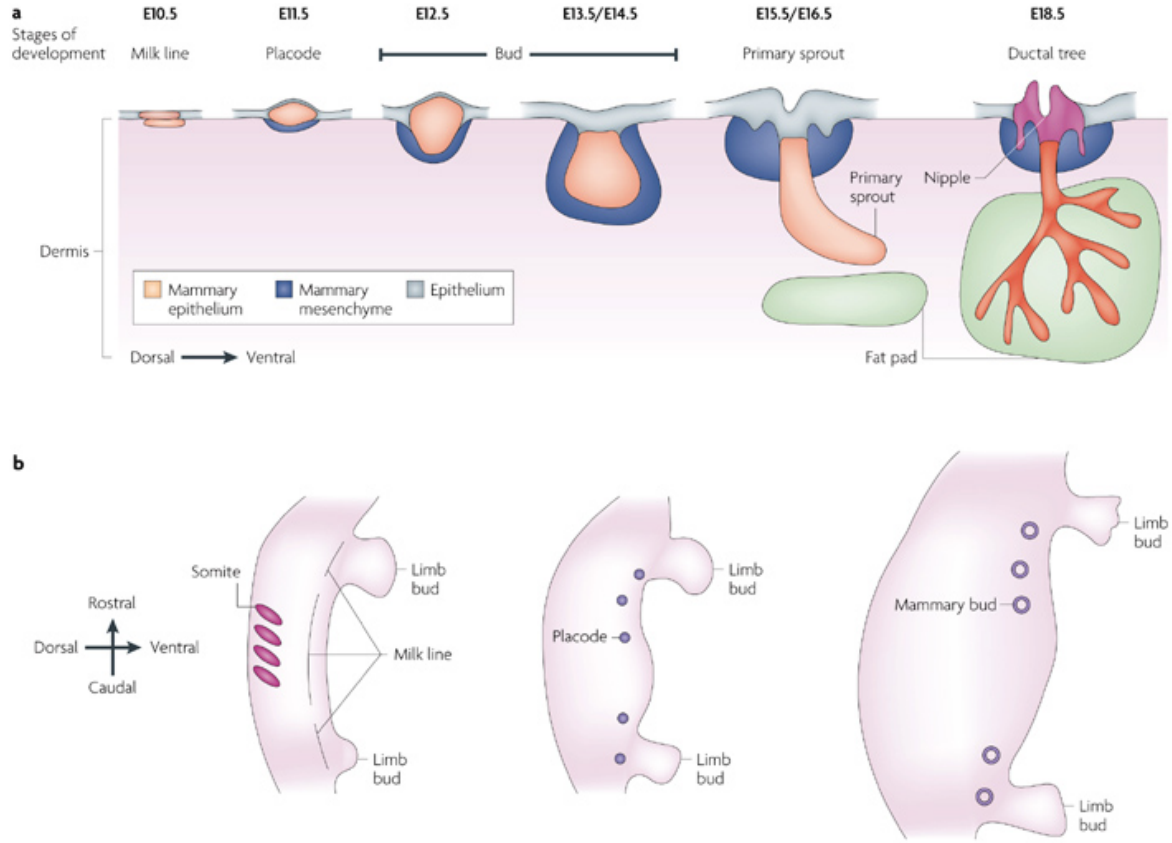


*Figure 2: (a) Morphological stages of the embryonic development of the mammary gland (b) the position of the milk line, placodes and mammary buds along the lateral body wall of early mouse embryos (Taken from (Robinson, 2007)).*

### 1.1.2 Postnatal mammary gland development

During the first three weeks of life, blood vessels in the fat pad are well visible. Elongation and branching of the mammary ducts take place slowly. Elongation of the ductal system initiates around the lymph node. The Terminal End Bud (TEB) represents the structure where elongation and branching of the duct takes place (Figure 3 a). The TEB of the mouse consists of two histological distinct cell types. The cap cells which are the precursors of myoepithelial cells, at the apex of the growing TEB are highly proliferative and differentiate into the myoepithelial cells which surround the mature duct (Humphreys, et al., 1996), (Figure 3 b,c). The body cells are the most prominent cells in the TEB and differentiate into the ductal epithelial cells (Figure 3 c).

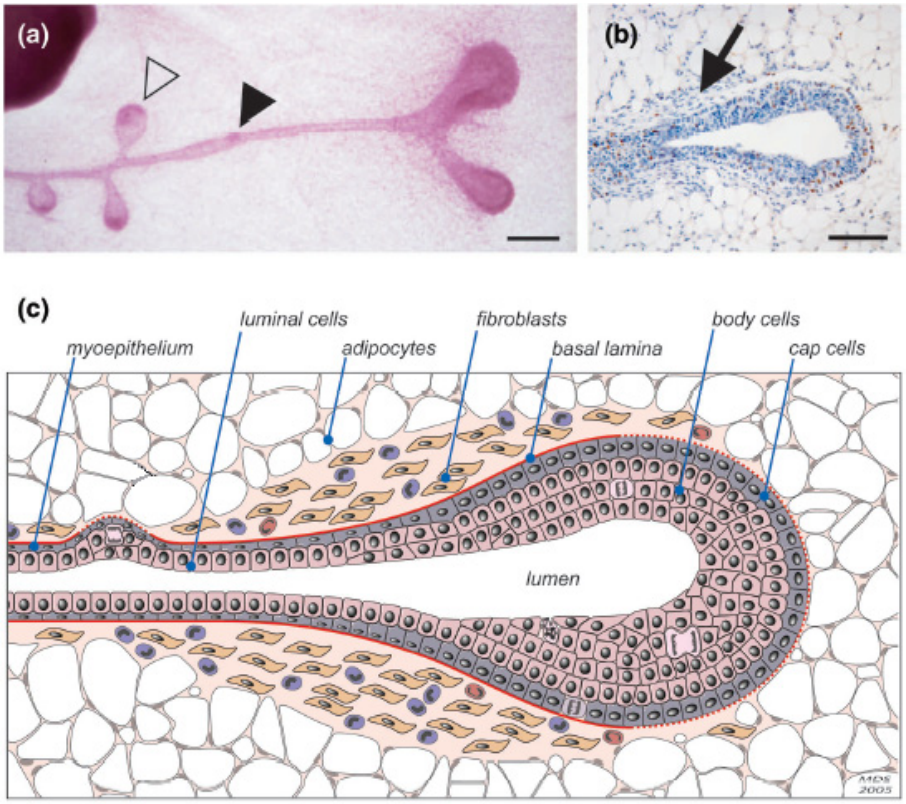


*Figure 3: Structure of the TEB and duct morphology; (a) primary duct (white arrowhead) and newly formed side branches (black arrowhead). (b) BrDu stained TEB exhibiting its considerable proliferative activity. (c) Schematic diagram depicting the salient architectural features of TEBs (Taken from (Sternlicht, 2006)).*

### 1.1.3 Mammary gland development during puberty and pregnancy

Puberty is the period of transition from childhood to adulthood. This process is regulated by several hormonal changes in the body. The period begins with release of follicle-stimulating hormone and lutenizing hormone from the anterior pituitary gland in a cyclic pattern. These hormones stimulate the ovaries to synthesize and release female sex steroid hormones, estrogen and progesterone. Estrogen stimulates the proliferation of the apical end of the branching structure known as TEB where the ductal elongation and branching takes place. However, estrogen does not adequately stimulate ductal growth by itself. Hereby, ductal growth into the adipose stroma to a branched epithelial network is executed by the alliance of several hormones such as prolactin and somototropin which can support alveolar development and subsequent milk production during pregnancy and lactation (Howlin, et al., 2006).

Mitotic activity remains very high until the ducts have reached the periphery of the mammary fat pad (Figure 3). At this point the TEB forms terminal ductal structures with low mitotic activity. Ductal development decreases with the acquirement of sexual maturity.

An increase in the number of epithelial cells may reflect a change in the size of these alveoli but also a change in the patterns of genes expressed in these cells (Rudolph, et al., 2007).

Two ovarian hormones, estrogen and progesterone are coordinating the expansion of the terminal ducts and alveologenesis (Brisken, et al., 1998), (Mallepell, et al., 2006).

Additionally, the expression of the three paracrine growth factors RankL, Wnt4 and amphiregulin in the mammary gland suggests that they might contribute to this process. Indeed amphiregulin expression is reduced in prolactin receptor knockout mice, suggesting that its expression might be regulated by prolactin (Ormandy, et al., 2003) and that it plays a role in alveogenesis during pregnancy, as indicated by other studies (Luetteke, et al., 1999).

The second half of pregnancy is characterized by the expansion of alveolar buds to form clusters of lobuloalveolar units. Alveolar differentiation is closely associated with the functional differentiation of the mammary epithelium termed lactogenesis. This process begins with increased and sustained expression of genes involved in the synthesis of different milk proteins such as Beta-casein, Alpha-lactalbumin and Whey acidic protein (WAP) (Neville, et al., 2002). These milk proteins are regulated by the prolactin signaling cascade.

The pituitary peptide hormone, prolactin plays a central role on administration of alveogenesis and lactogenesis (Brisken, et al., 1999). Prolactin also induces many transcription factors including Sterol Regulatory Element Binding Factor-1 (SREBF-1) that regulates the expression of a number of key lipid metabolism genes (Naylor, et al., 2005). At the middle of pregnancy size and cellular distribution of lipid droplets in the epithelial cells considerably increase (Figure 4 a,b,c). Lipid droplets provide a convenient system for delivering large amounts of energy to the suckling neonate because, unlike small molecules such as lactose, they exert little osmotic pressure and they can be stored in large quantities in alveolar spaces following secretion. These cytoplasmic lipid droplets (CLD) can be seen within luminal mammary epithelial cells. Following parturition, CLDs are smaller and localized to the apical surface of the alveolar epithelial cells and disappear after lactation day 2 (Figure 4 d).

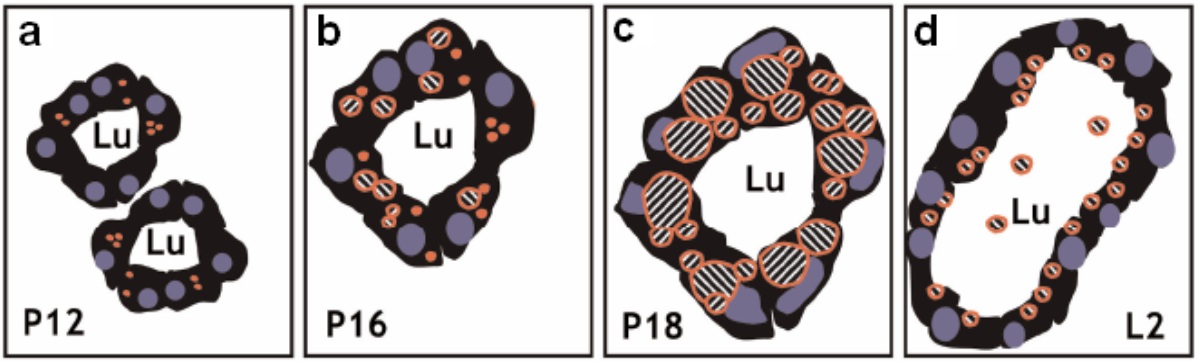


*Figure 4: Illustrated structures of alveoli during pregnancy (P) days 12 (a), 16 (b), 18 (c) and lactation day 2 (d) Lu: luminal space, nuclei: labelled blue , CLD: labelled red (Taken from (Anderson, et al., 2007).*

### 1.1.4 Mammary gland development during lactation period

Continuous milk production and intermittent secretory activity is the physiological function of the mammary gland which is termed lactation. The lactating mammary gland is composed of a branching network of ducts formed of epithelial cells ending in extensive lobuloalveolar clusters. An alveolus is surrounded by a monolayer of polarized secretory epithelial cells which are enclosed by myoepithelial cells that function in milk ejection and a vascularized connective tissue that contains lipid depleted adipocytes and fibroblasts (Figure 5). After the parturition, a rapid series of changes in the activity of differentiated mammary epithelial cells from a quiescent state to a fully active secretory state occur. Following the parturition, the level of the ovarian hormone progesterone decreases and the level of the pituitary hormone prolactin increases (Neville, et al., 2002). Elevation of the prolactin level leads to maximal activation of Signal Transducer and Activator of Transcription 5 (STAT5) and increases the transcription level of milk protein genes such as caseins, lactalbumins and WAP (Muldoon, 1987), (Robinson, et al., 1998).

By virtue of high secretory activation, the cytoplasm of the lactating alveolar cells contains numerous mitochondria, extensive rough endoplasmic reticulum and a well-developed golgi network. One of the special features of this complex is the synthesis of lactose from UDP-galactose and glucose with the presence of high concentration lactose phosphate, calcium and citrate (Clermont, et al., 1993). D-glucose is essential for the secretion of milk volume involving the precursor of lactose and cellular energy source (Linzell; Peaker, 1971). Uptake of the glucose is mediated by the GLUT family of genes which are specialized glucose transport proteins within the mammary epithelial cells.

Lactose, milk proteins such as Beta-casein, Alpha-lactalbumin, WAP and important substances such as phosphate, calcium and citrate are packaged into secretory vesicles and are transported to the apical region of the alveolar cells. Connectivity of the epithelial cells to the neighbouring cells is ensured through a complex composed of adherents and tight-junctional elements that ensure the transport of substances between basal area and epithelial cells (Brisken, et al., 1998), (Robinson, et al., 2000). After parturition, leaky tight junction complexes evolve into impermeable walls. This functional shift of tight junction plays an important role in lactation by preventing the loss of milk components from alveolar lumen to the basal area. Closure of the tight junctions is accompanied with many conditions including milk secretion (Stelwagen, et al., 1997), (Nguyen, et al., 2001) and decreased progesterone level (Neville, et al., 2002). Physiologically this process is termed as secretory activation.

In addition to lactose synthesis, the lactating epithelial cells have very special synthetic capabilities for lipid synthesis. Milk lipids, primarily triacylglycerides, are synthesized in the smooth endoplasmic reticulum in the basal region of the alveolar cell composed of a glycerol

backbone and esterified fatty acids, which are either derived from the diet, from adipose tissue stores, or synthesized *de novo* in the gland. Newly synthesized lipid molecules form into small protein coated storage structures termed CLD. Secretory vesicles containing CLDs (Mather; Keenan, 1998), (Capecchi, 2005) and casein move to the apical region of the cell to be secreted as a membrane bound milk fat globule (Jensen, et al., 2001), (McManaman; Neville, 2003).

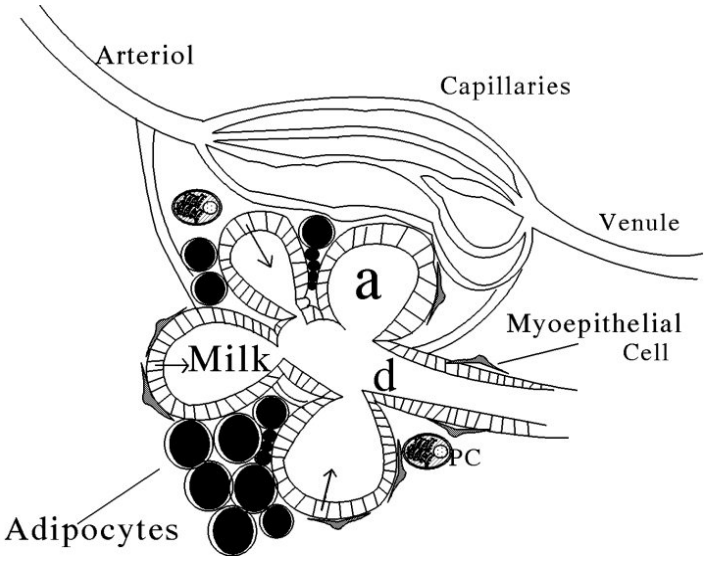


Figure 5: Model alveolus (a) with subtending duct (d) showing blood supply, adipocyte stroma, myoepithelial cells, and plasma cells (PC) (Taken from(McManaman; Neville, 2003)).

Synthesized milk is stored in the alveolar lumen that is surrounded with myoepithelial cells (Figure 5 a) and drains through ductules into ducts. These ducts coalesce into main ducts that drain sectors of the gland. The main ducts dilate into small sinuses as they near the areola where they open directly on the nipple. In many species including both ruminants and rodents the ducts empty into a single primary duct or a cistern which in turn is drained by a single teat canal (McManaman; Neville, 2003).

Removal of milk from alveoli to the suckling pups is termed milk ejection. This process begins with the suckling impulse that promotes the release of oxytocin from the posterior pituitary. The release of oxytocin is necessary for the maintenance of lactation (Nishimori, et al., 1996) and modulation of the prolactin release as positive feedback (Juszczak; Stempniak, 1997). The oxytocin is carried through the blood flow to the mammary gland where it interacts with specific receptors on myoepithelial cells, and induces contraction of myoepithelial cells. Contraction of myoepithelial cells charges the milk flow from alveolar lumen to the nipple.

#### 1.1.4.1 Milk and major milk proteins

Milk is a balanced mixture of nutrients for neonatal growth released by the mammary gland during the lactation period. The general fundament of the milk is composed of milk proteins, immunoglobin A, lactose, monovalent ions and fat. The composition of these ingredients exhibits variations depending on the species. The expression of milk proteins is closely intertwined with the functional differentiation of the mammary epithelium. Therefore the gene expression patterns vary slightly between caseins and whey proteins. It has been noted that the casein transcription increases rather early during pregnancy, whereas high levels of expression of the WAP and Alpha-lactalbumin are restricted to the last phase of pregnancy in mice (Pittius, et al., 1988), (Robinson, et al., 1995).

#### 1.1.4.2 Alpha-lactalbumin

Alpha-lactalbumin, known as a milk protein functions also as an essential cofactor of galactosyl transferase to compose lactose syntase that catalyse a special reaction to synthesis of lactose from glucose (Neville, 2009). Therefore it has a main function in terms of control of milk secretion (Brew, et al., 1968). It was noted that Alpha-lactalbumin knockout mice were unable to nourish their pups and this defect is defined as milk stasis due to the incapacity to eject highly viscous and fatty milk (Stinnakre, et al., 1994). Its very low gene expression during pregnancy is increased after the parturition and throughout the lactation period (Rudolph, et al., 2007).

#### 1.1.4.3 Beta-casein

Caseins are a family of proteins, which incorporate inorganic phosphate from the blood. Due to the high proline content the caseins have hydrophobic characters which are found in milk as suspension of particles termed casein micelles (Dalgleish, 1998 ). These colloidal micelles play an important role in cellular uptake of ions such as calcium and phosphate through soluble assemblies (O'Connell, et al., 2003) and are hypothesized to prevent protein aggregation (Morgan, et al., 2005).

Beta-casein is a transcriptional target of STAT5 downstream of the prolactin receptor signaling pathway (Liu, et al., 1997). Moreover, STAT5 transgenic mice represented higher levels of Beta-casein secretion into the milk which is accompanied by enhanced growth of pups (Iavnilovitch, et al., 2002). In line with the possession of lactogenic hormones on the regulation of milk proteins; the expression of the Beta-casein gene increases over the course of pregnancy and then undergoes a further increase at secretory activation and dramatically decreases at the onset of involution. A sudden downregulation of Beta-casein expressions has been noted when the secretory activation had not occurred after parturition (Watkin, et al., 2008).

#### 1.1.4.4 Whey acidic protein (WAP)

WAP maintains the stability of secretory proteins in milk as a dispensable protease inhibitor (Wilde, et al., 1995), (Triplett, et al., 2005). The expression of the WAP gene is principally restricted to the mammary gland. In fact its expression is increased by a variety of lactogenic hormones such as prolactin (Doppler, et al., 1991) and glucocorticoids (Vonderhaar; Ziska, 1989) and partially dependent on STAT5 activity (Inuzuka, et al., 1999). Therefore transcriptional regulation of the WAP gene occurs during pregnancy and lactation.

Numerous studies on the transcriptional regulation of this major milk protein have been linked to functional differentiation of mammary epithelial cells during pregnancy. In fact, the expression of WAP has been considered as an advanced differentiation marker (Brandt, et al., 2000). However WAP depleted mice did not exhibited striking phenotypic abnormalities. This suggests that the expression of the WAP gene is not required for alveolar specification and functional differentiation (Triplett, et al., 2005). Nevertheless, in line with its essential function female mice of this animal model were not able to nourish adequately their growing young (Triplett, et al., 2005).

Beside its function in mammary gland physiology, WAP has been recognized as a suppressor of the proliferation of mammary tumour cells by a paracrine mechanism (Nukumi, et al., 2007). Consequently, WAP has been addressed as a therapeutic protein against the development of breast cancer and tumourigenesis (Nukumi, et al., 2007).

### 1.1.5 Involution of the mammary gland

Mammary gland involution is defined as the transition of the lactating gland to the non-lactating stage in preparation for a subsequent pregnancy. The involution process is initiated after cessation of milking, sudden weaning of the offspring or gradual decrease in suckling. During involution, the milk producing lobuloalveolar structures are regressed to a simple ductal tree formation.

Absence of suckling stimuli or milk stasis results in a rapid induction of local factors, leading to apoptosis of the alveolar epithelium (Figure 6, 1st phase) and consequently to decline in circulating lactogenic hormone concentrations. Milk stasis induces the expression of leukaemia inhibitory factor (LIF) and transforming growth factor (TGF) β3 within 12 hours of forced weaning (Green; Streuli, 2004), (Watson, 2006). LIF is a cytokine that activates Signal Transducer and Activator of Transcription 3 (STAT3) which is critical for the initiation of apoptosis and involution (Kritikou, et al., 2003), (Chapman, et al., 1999).

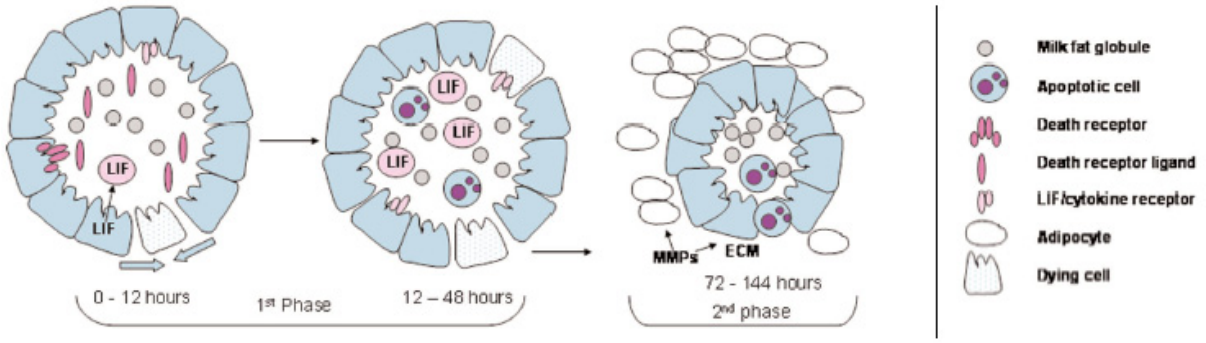


*Figure 6: Processing of involution (Taken from (Watson, 2006)).*

In response to STAT3 activation the nucleus and cytoplasm condense, the chromatin becomes fragmented and apoptotic cells are scattered into the alveolar lumen (Strange, et al., 1992). This cell loss results in extensive disintegration of the alveolar structure during the first stage of involution in the mouse, but it is a reversible process which is suppressed or elongated by various factors (Schwertfeger, et al., 2001), (Fata, et al., 2000).

During the second stage of involution alveoli collapse and adipocytes furnish the remaining area. Regulation of this process is controlled by the activation of Matrix metalloproteases (MMP) and serine proteases. The MMPs are primarily expressed by the stromal cells and are upregulated (MMP2, MMP3 and MMP9) during involution. By the activation of MMPs, reversibility of the first phase is lost (Fata, et al., 2000)and the tissue remodelling process begins. Active MMPs demolish the extracellular matrix (Figure 6 ECM) which is surrounding each alveolus, resulting in detachment induced apoptosis and collapse of the alveoli. By the inflammatory cell infiltration of the mammary gland, large numbers of cells and debris are removed by phagocytosis (Monks, et al., 2005). Following the tissue remodelling, adipocytes are re-differentiated by action of MMP3 and MMP regulated plasminogen (Lund, et al., 2000) and the mammary gland returns to the pre-pregnant state.

## 1.2 Components of the endothelin system

Endothelin, an important substance in the maintenance of vascular tone, was firstly isolated and characterised by Yanagisawa from cultured supernatant of porcine aortic endothelial cells. Following the isolation of the endothelin (ET-1) cDNA, corresponding to amino acid residues 7-20 of ET-1, two more isomers were identified and referred to endothelin-2 (ET-2), and endothelin-3 (ET-3), (Yanagisawa, et al., 1988), (Inoue, et al., 1989).

ET-1, ET-2 and ET-3 are located on chromosome 6, 1 and 20, respectively (Heyl, et al., 1993), (Nakajima, et al., 1989). The dispersal of these three preproendothelin genes indicates that they are not genetically related. The presence of ET-like sequences in each of these genes, suggests that a common ancestral exon was duplicated to generate a highly homologous ET-like sequence within each gene structure.

ET-1 is expressed as an inactive precursor peptide comprising 212 amino acid residues, named preproendothelin. Two endopeptidases control the activation process of preproendothelin (Itoh, et al., 1989). Firstly, the prepro form of endothelin is cleaved by a dibasic endopeptidase at the lys51-arg52 and arg 91-arg 92 position to form big ET-1 or proET-1 which is biological inactive (Figure 7).

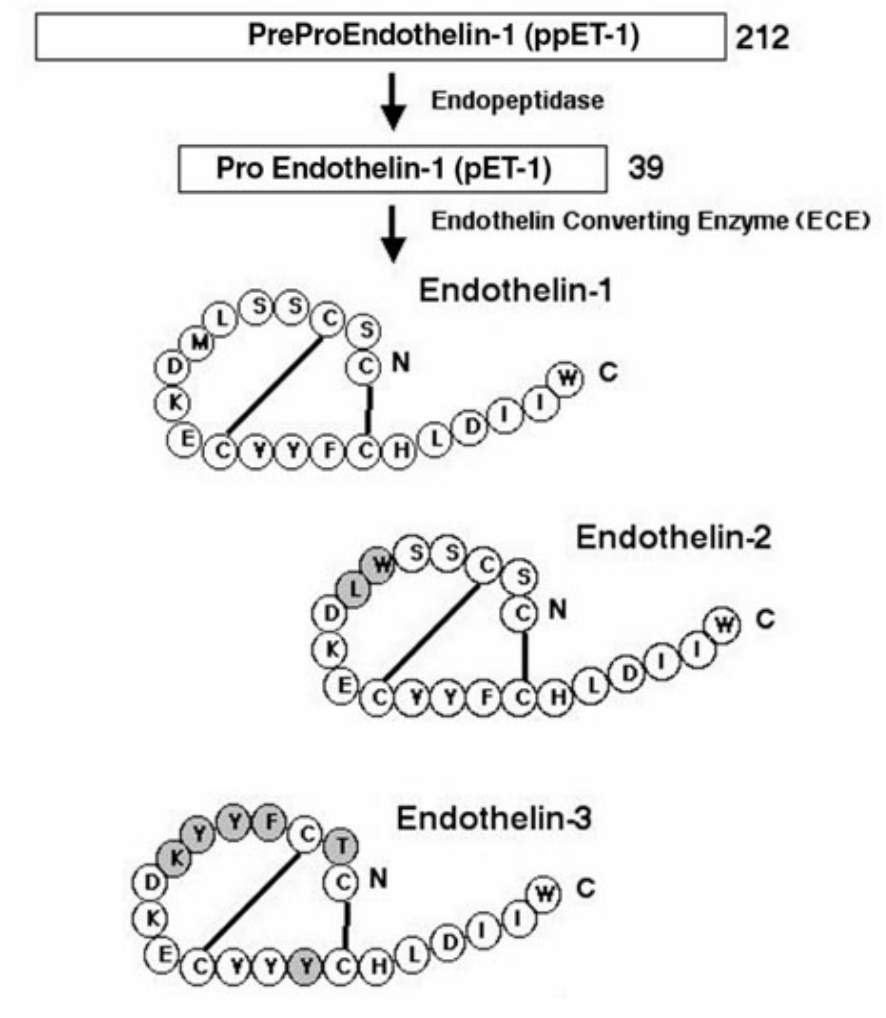


*Figure 7: Biosynthesis and amino acid sequence and structure of endothelin-1, endothelin-2, and endothelin-3. ET-2 and ET-3 differ from ET-1 by two and five amino acids, respectively.*

Secondly, the conversion of proET-1 to the biologically active ET-1 is catalysed by a furin like protease named Endothelin Converting Enzyme (ECE). The cleavage occurs in the intracellular compartments and on the cell surfaces and leads to the active ET-1. The active ET-1 comprises 21 amino acids and is folded by two disulphide bridges between 1-15 and 3-11 designating the half life of the biological activity (Takaoka, et al., 1990). The half life of ET-1 in vivo is less than one minute whereas pro ET-1 lasts approx. 20-25 min (Levin, 1995). Therefore, under normal physiological conditions ET-1 is not a circulating hormone but rather ET-1 functions in an autocrine or paracrine manner at multiple locations in the body (Battistini, et al., 1993); (Moraitis, et al., 1999).

ET-1 is the predominant and most extensively studied isoform of the endothelins which is mainly synthesized in endothelial cells, vascular smooth muscle cells and heart (Yanagisawa, et al., 1988), lung (MacCumber, et al., 1989), kidney (Karet; Davenport, 1996), brain (Giaid, et al., 1991), macrophage cells (Ehrenreich, et al., 1990) and mammary gland (Kozakai, et al., 2002).

Regulation of ET-1 arises particularly on the transcriptional level because the cells that produce ET-1 do not have storage vesicle or a regulated secretory pathway. A large number of factors effect ET-1 expression, for instance, Transforming Growth Factor β (Kurihara, et al., 1989), Tumor Necrosis Factor α (Marsden; Brenner, 1992), interleukin 6 (Woods, et al., 1999), glucocorticoids (Morin, et al., 1998), angiotensin II (Kohno, et al., 1992), and thrombin (Emori, et al., 1992) have been demonstrated to upregulate ET-1 expression in a variety of cells. Furthermore, physical factors such as shear stress and stretch (Malek, et al., 1999), and hypocapnia (Yoshimoto, et al., 1991) increase the level of ET-1 expression. In contrast, nitric oxide (Boulanger; Luscher, 1990), prostacyclin (Prins, et al., 1994) and atrial natriuretic hormone (Kohno, et al., 1992) downregulate ET-1 expression in endothelial cells. In addition to the physiological complexity of the endothelin system, distribution of other endothelin system components exhibit variations (Table 1), (Kedzierski; Yanagisawa, 2001).

*Table 1: Expression pattern of components of the endothelin system in normal physiology; (*) The ET-1 expression occurs during pregnancy and lactation.*

| | **ET-1** | **ETAR** | **ETBR** |
|---|---|---|---|
| Endothelial cells | **+** | | **+** |
| Smooth muscle cells | | **+** | **+** |
| Cardiomyocytes | **+** | **+** | **+** |
| Hepatocytes | **+** | **+** | **+** |
| Renal cells | **+** | | **+** |
| Neurons | **+** | **+** | **+** |
| Osteoblasts | | **+** | **+** |
| Keratinocytes | **+** | **+** | **+** |
| Adipocytes | | **+** | **+** |
| Mammary epithelium | **+(*)** | **+** | **+** |

Beside its role as a potent vasoconstrictor in the cardiovascular system, ET-1 has been involved in numerous physiological processes including renal volume homeostasis, wound healing (Papalambros, et al., 2008), intestinal tract movement (Kernochan, et al., 2002), control of menstruation (Salamonsen, et al., 1999), neural crest cell development (Kurihara, et al., 1994) and regulation of airway tone in the lung (Adamicza, et al., 1999) .

ET-1 exerts its effect by binding to two distinct G protein-coupled receptors, the Endothelin A (ETAR) and B Receptor (ETBR). The ETAR has a subnanomolar affinity for ET-1 whereas its affinity to the other isoforms is about 2 orders of magnitude lower. In contrast, the ETBR has equal subnanomolar affinities for all endothelin isoforms (Arai, et al., 1990), (Sakurai, et al., 1990). On the functional level both receptors differ, ETAR predominantly mediates vasoconstriction in vascular smooth muscle cells (Marsault, et al., 1993) whereas, ETBR activation results in vasodilatation in vascular endothelial cells. (Yanagisawa; Masaki, 1989). Beside the function in the vascular system, the ETAR has been involved in the regulation of mitogenesis and furthermore appears to have anti-apoptotic effects (Nelson, et al., 2003). ETBR activation has been associated with ET clearance and angiogenesis.

As GPCRs, both the ETAR and ETBR exert their effect by the recruitment of second messenger systems such as serine threonine kinases and ion channels (see chaper 1.2.2 GPCRs).

### 1.2.1 Animal models and the study of the endothelin system

Several strategies were employed to understand the functional relevance of the ET system in physiology and pathophysiology (Hocher; Paul, 2000). In principal, two concepts have been used in recent years: first, a gene knock out model was generated to study the functional importance of ET-1. The loss of function of ET-1 caused death in newborns due to craniopharyngeal malformations resulting in an inability to breathe after birth (Kurihara et al. 1994). ETAR knock out mice exhibited the same phenotype (Abe, et al., 2007). On the other hand, the major phenotype in ETBR deficient mice was the absence of enteric ganglion cells, leading to a congenital megacolon with early postnatal death from Hirschsprung's disease (Hosoda, et al., 1994). In a second approach Theuring and coworkers engineered transgenic mice expressing a human genomic ET-1 construct containing some 5' and 3' promoter sequences (Hocher, et al., 1997). Assessment of the transgene expression facilitates a valid model to elucidate functional consequences of the ET-1 overexpression in physiological and pathophysiological conditions in vivo.

Investigations on ET-1 transgenic mice demonstrated the importance of the endothelin system in different tissues and organ functions. Augmented ET-1 expression and its components had been associated with diverse pathological human conditions, including hypertension (Rothermund; Paul, 1998), cardiac failure, kidney diseases (Kohan, 1997) and various solid tumours such as hepatocellular, ovary and breast (Nakamuta, et al., 1993), (Bagnato, et al., 1999), (Yamashita, et al., 1991).

### 1.2.2 G protein-coupled receptors (GPCRs)

GPCRs represent the largest family of cell-surface receptors (Hermans, 2003) involved in the regulation of numerous physiological functions including neurotransmission, photoreception, chemoreception, metabolism, growth and differentiation (Fukuhara, et al., 2001).

GPCRs are also called heptahelical or serpentine receptors as they contain a conserved structural motif of seven α-helical membrane-spanning regions. N-terminal segments, the cyto- and exoloops as well as the C-terminal segment can greatly vary in size. The amino-terminus is exposed to the extracellular environment. The C-terminus and the intracellular loops interact with intracellular signaling partners, such as the associated heterotrimeric G proteins, but also with a wide variety of proteins containing structural interacting domains (Bockaert; Pin, 1999).

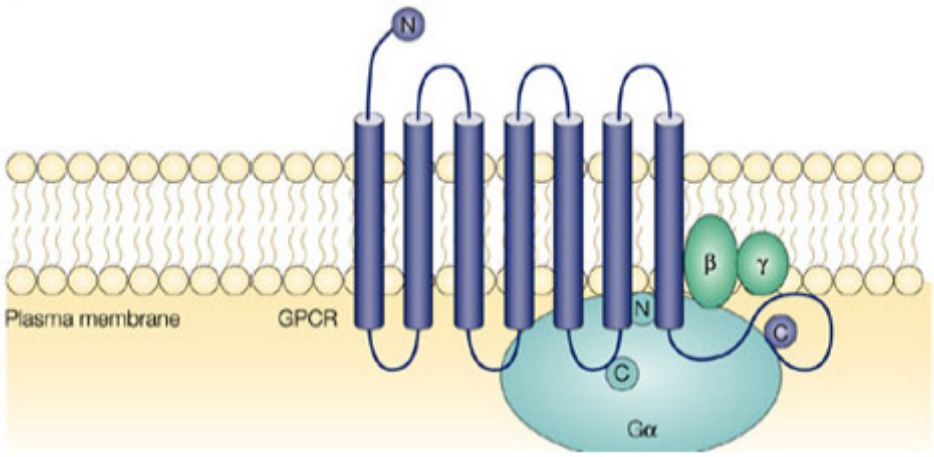


*Figure 8: Schematic drawing of a G protein-coupled receptor (GPCR) G∝ fusion protein. (Taken from (Ellis, 2004 ).*

A common biochemical characteristic of GPCRs is their interaction and activation of down-stream signaling cascades by heterotrimeric G proteins. Heterotrimeric G proteins are composed of a Gα subunit interacting with a Gβγ subunit (Figure 8). Ligand binding to GPCRs alters the conformation of intracellular receptor domains and induces the association with heterotrimeric G proteins. This results in an exchange of GDP for GTP in the active site of the Gα subunit, followed by the dissociation of the heterotrimeric complex. Both the Gα and Gβγ subunits activate cytoplasmic or membrane bound effector molecules (Hermans, 2003).

G proteins are generally referred to their Gα-subunits. At least 23 different mammalian G protein α subunits have been cloned and are divided into four distinct families based on amino acid sequence similarity, namely $G\alpha_s$, $G\alpha_q$, Gαi and $G\alpha_{12/13}$. Similarly, 12 Gγ subunits and 6 Gβ subunits have been identified (Hermans, 2003), (Pierce, et al., 2001).

Once Gα-GTP has dissociated from the Gβγ-dimer, Gα can directly interact with effector proteins such as $Ca^+$ and PKC to continue the signaling cascade. Overall, several patterns

emerge upon examination of the Gα effectors. Each Gα family activates a distinct profile of effectors. The Gαs protein is activated by GPCR (Figure 9 GPCR1), which leads to at least two downstream responses: firstly, the cyclic AMP (cAMP) is generated by adenylyl cyclase (AC) and subsequently protein kinase A (PKA) is activated, which, through its catalytic subunits (C), can phosphorylate nearby substrates (Berlot; Bourne, 1992). Secondly, the mitogen-activated protein kinase (MAPK) cascade is activated by the mediation of Gβγ-complex, which results in the activation of extracellular signal-regulated kinase-1/2 (ERK1,2), which mediates transcriptional responses (Osmond, et al., 2005).

Among the first GPCR recognized to have growth transduction effects were those that interact with $G\alpha_q$. Activation of $G\alpha_q$ (Figure 9 GPCR2) and therefore activation of phospholipase Cβ (PLC β) generates water-soluble inositol-1,4,5-triphosphate ($InsP_3$) and diacylglycerol (DAG) from phosphatidylinositol-4,5-bisphosphate ($PtdIns(4,5)P_2$). $InsP_3$ regulates the mobilization of intracellular $Ca^{2+}$ ions ($[Ca^{2+}]_i$) and the activation of $Ca^{2+}$/calmodulin-activated protein kinase II (CaMKII), whereas DAG activates protein kinase C (PKC). Both CaMKII and PKC can phosphorylate nearby substrates (Chidiac; Ross, 1999).

Several mitogenic hormones and ligands including LPA, thrombin, endothelin and bombesin interact with receptors that are linked to both $G\alpha_q$ and $G\alpha_i$, (Daaka, 2004). GPCR activates $G\alpha_i$, which induces two downstream signalling events, the Gβγ-complex-mediated activation of PLC β and the activation of phosphodiesterase-6 (PDE6). PDE6 reduces the intracellular concentrations of cyclic GMP, protein kinase G (PKG) activity and cyclic-nucleotide-gated channel (CNGC) activity by three parameters (Figure 9 GPCR3).

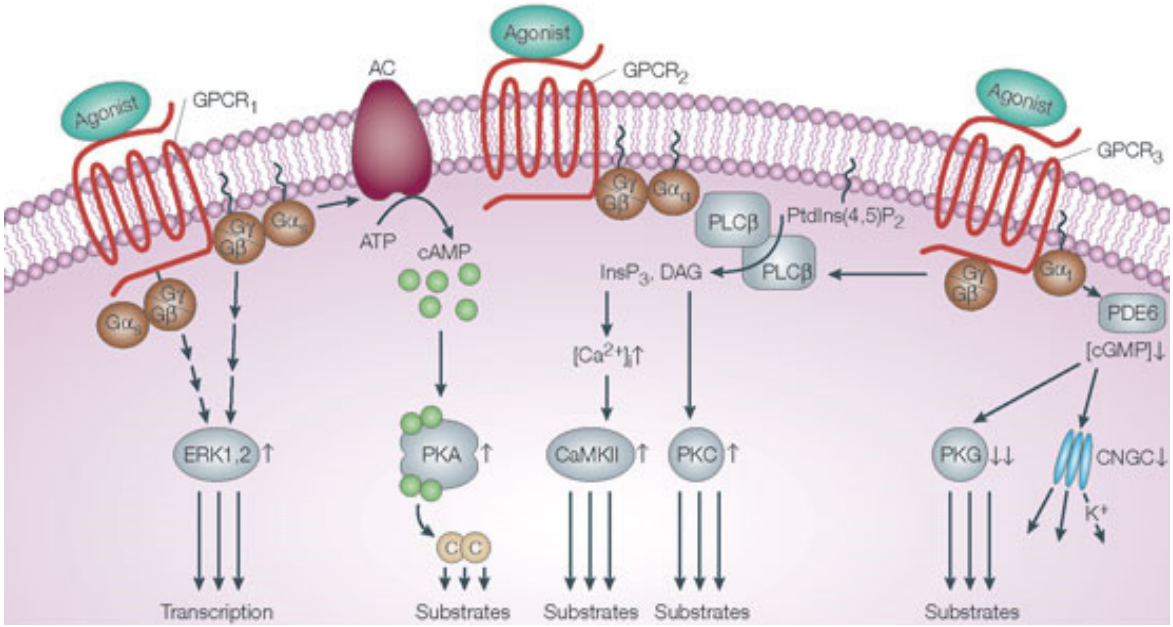


*Figure 9: Downstream responses of GPCRs within their Gα-subunits, (Taken from (Malbon, 2005)).*

$G\alpha_{12/13}$ subunits constitute a family of G proteins distantly related to other G proteins; both are activated by the thrombin receptor in platelets (Offermanns, et al., 1994). Delineation of effectors involved in nuclear signalling by these G proteins is the focus of much ongoing research.

Apart from Gα subunits, initially, Gβγ was thought to facilitate the completion of intracellular information transfer passively by binding to Gα and hastening the return of the heterotrimer to the plasma membrane, thereby preventing spontaneous Gα activation in the absence of receptor stimulation. Today, Gβγ is known to activate and interact with several effectors including PLC β (Sternweis, 1994), AC (Tang; Gilman, 1991), $PI_3$ kinase (Tang; Downes, 1997) of $K^+$ and $Ca^{2+}$ selective ion channel (Herlitze, et al., 1996). Therefore, GPCRs are likely to represent the most diverse signal transduction systems in eukaryotic cells.

## 1.3 Epidermal growth factor receptor (EGFR)

One of the most important tyrosine kinase signaling networks is a group of receptors belonging to the "HER" family, also known as the ErbB signaling network. The ErbB receptors are named after the avian erythroblastosis tumor virus, which encodes an aberrant form of the human epidermal growth factor receptor (from which "HER" originates).

The HER family of receptors which consists of four closely related receptor tyrosine kinase genes are commonly referred to as HER1/EGFR, HER2, HER3 and HER4 (Ullrich; Schlessinger, 1990). Each receptor contains an extracellular ligand-binding domain, a transmembrane lipophilic domain and an intracellular tyrosine kinase domain (Figure 10 a).

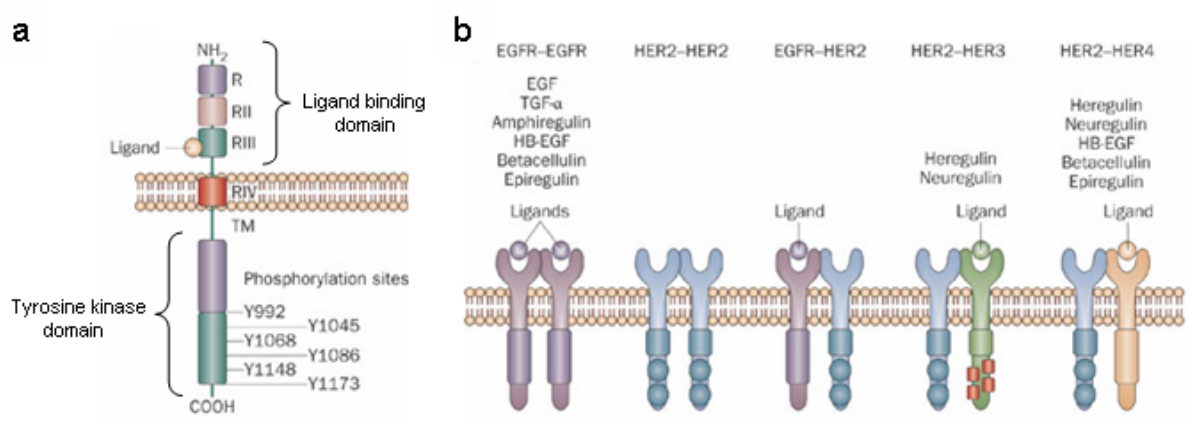


*Figure 10: The HER family of tyrosine kinase receptors. No ligand for HER2 has been identified. Ligand binding induces autophosphorylation and causes activation of downstream signaling pathways. Ligands are indicated by circles, PI3K binding sites are indicated by red squares (Taken from (Linardou, et al., 2009)).*

The ligand binds the extracellular ligand-binding domain of its cognate receptor and mediates receptor dimerisation, either homodimerisation such as EGFR-EGFR (Figure 10b) or heterodimerisation such as EGFR-HER4 (Figure 10b) with a neighbouring activated receptor molecule (Garrett, et al., 2002), (Ogiso, et al., 2002).

Dimerization of the receptor induces a conformational change in the cytosolic part of the receptor thereby generating docking sites for intracellular signal transducers with phosphotyrosine interaction domains (Figure 10a), (Schlessinger, 2002).

Tyrosine phosphorylation of the EGFR leads to the recruitment of diverse signaling proteins, including the adaptor proteins GRB2 (Growth Factor Receptor-Bound Protein-2) and Nck (Nck Adaptor Protein), PLC-Gamma (Phospholipase-C-Gamma), SHC (Src Homology-2 Domain Containing Transforming Protein), leading to the activation of downstream signaling cascades including the RAS/ERK pathway, the PI3/AKT pathway, the JAK/ STAT pathway, and several other proteins and molecules (Figure 11).

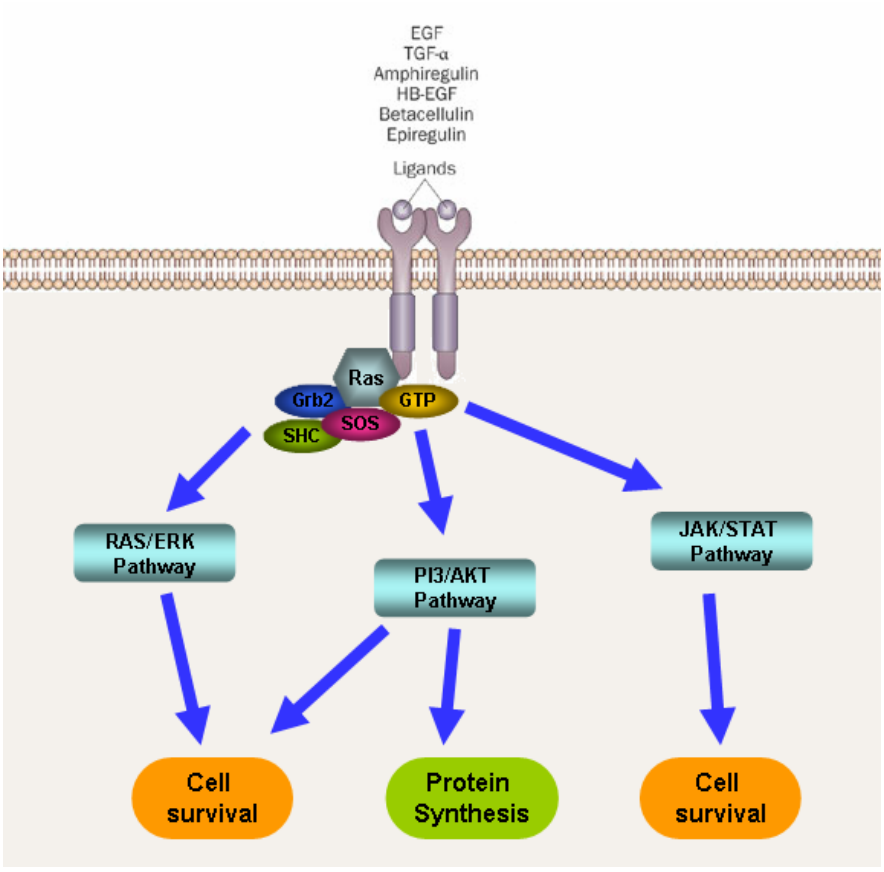


*Figure 11: Activated EGFR results in the activation of most widely known and best characterised signaling cascades. RAS/ERK pathway, PI3/AKT pathway and JAK/STAT pathway (According to (Linardou, et al., 2009)).*

The EGFR is the most prominent receptor tyrosine kinase (RTK) and is ubiquitously expressed in nonhemopoetic tissues. Thus EGFR activation is positioned to affect a wide range

of cellular responses, depending on the coordinate expression of the cognate ligand (Peles; Yarden, 1993).

Eight ligands are known to directly activate the EGFR: EGF (Cohen, 1986), TGFa (Luetteke; Lee, 1990), heparin binding EGF (Higashiyama, et al., 1992), amphiregulin (Shoyab, et al., 1988), betacellulin, epiregulin (Riese, et al., 1998), epigen (Strachan, et al., 2001) and cripto (Salomon, et al., 1999). Each of these molecules except cripto is synthesized as a transmembrane precursor and requires proteolytic cleavage of its ectodomain to produce a soluble growth factor which activates receptors of the EGFR family by autocrine or paracrine stimulation (Riese, et al., 1998).

The proteolytic processing of EGF ligands is achieved by metalloproteases of the ADAM subfamily (A Disintegrin and Metalloprotease). Control of the cleavage is based on sensitivity of the GPCR agonist or tyrosine kinase stimulation (Massague; Pandiella, 1993), which leads to a paracrine growth and survival factors including EGFR ligands allowing a consistent supply of EGFR agonists (Schlondorff; Blobel, 1999).

Genetically modified animal models disclose the importance of EGFR in development. For instance, absence of the EGFR leads to embryonic or perinatal death with multiple organ abnormalities in mice (Sibilia; Wagner, 1995), (Threadgill, et al., 1995).

Loss of the EGFR activity in waved-2 mutant mice exhibit impaired ductal outgrowth (Fowler, et al., 1995), (Xie, et al., 1997) in the mammary glands. In addition, transplantation of mammary glands derived from EGFR knockout mice exhibited impaired ductal development in the host mice (Wiesen, et al., 1999). These studies indicate that EGFR is essential for ductal development.

The importance of the EGFR is also linked to its ligands which can influence the mammary gland development. Indeed, ductal outgrowth is severely impaired in triple-null mice lacking amphiregulin, EGF and TGFa which are lactation incompetent and variably impaired compared to mice lacking only amphiregulin (Luetteke, et al., 1999). Elimination of EGF, TGFa, HB-EGF and betacellulin alone or in various combinations affect neither ductal outgrowth nor lactation (Jackson, et al., 2003), (Luetteke, et al., 1999). Amphiregulin is the sole required EGFR ligand for post-pubertal mammary development. Therefore, amphiregulin is strongly upregulated at puberty and dramatically downregulated during and after pregnancy (D'Cruz, et al., 2002), (Schroeder; Lee, 1998).

### 1.3.1 Amphiregulin

The EGFR ligand amphiregulin shares 38% amino acid sequence homology with EGF and 32% with TGFa (Shoyab, et al., 1989). Amphiregulin was originally identified from 12-O-tetradecanoyl-phorbol-13-acetate (TPA) conditioned medium of the human breast carcinoma

cell line MCF-7 (Shoyab, et al., 1988). Expression of amphiregulin emerged in a variety of carcinoma cell lines and in non-transformed epithelial and mesenchymal cells from the colon, stomach, lung, breast, ovary kidney and breast (Plowman, et al., 1990).

Amphiregulin is a bifunctional growth factor that promotes proliferation and survival (Lorente, et al., 2009) or apoptosis (Shoyab, et al., 1988), (Johnson, et al., 1992) depending on its concentration, presence of other EGFR ligands and the nature of the target cells.

Amphiregulin is expressed in the mammary gland as a primary paracrine regulator of estrogen induced ductal morphogenesis (LaMarca; Rosen, 2007). However, amphiregulin is only expressed in mammary epithelial cells but not in mammary stromal cells (Sternlicht, et al., 2005). The frequency and levels of the protein expression of amphiregulin are ordinarily higher in invasive breast carcinomas than in ductal carcinomas in situ (Salomon, et al., 1995).

Amphiregulin is regulated by estrogen (Kenney, et al., 1993), (Ciarloni, et al., 2007), TPA (Martinez-Lacaci, et al., 1996) and LIF (Sherwin, et al., 2004) by diverse mechanisms. Amphiregulin expression is also reduced in prolactin receptor knockout mice. This evidence indicates that amphiregulin might be regulated by prolactin in mammary epithelial cells during pregnancy (Ormandy, et al., 2003).

On the other hand, the activation mechanism of amphiregulin through cleavage by ADAM 17 (Sahin, et al., 2004), (Kenny; Bissell, 2007) is achieved in response to agonists acting through GPCRs in a process termed EGFR transactivation (Gschwind, et al., 2003), (Schafer, et al., 2004), (Kasina, et al., 2009).

### 1.3.2 EGFR transactivation by GPCR

A number of reports have demonstrated that various extracellular stimuli, unrelated to EGF-like ligands such as phosphatidic acid (LPA) (van Biesen, et al., 1995), ET-1 (Cazaubon, et al., 1994), bradykinin (Lev, et al., 1995) or thrombin (Chen, et al., 1996) results in activation of ERK1/2 and induction of *c-fos* gene expression as EGFR characteristic intracellular signals.

Furthermore, Daub and co-workers recognized the engagement of GPCRs and the EGFR as an essential signaling network for mitogenesis of rat fibroblasts (Daub, et al., 1996) This novel signaling network is termed receptor crosstalk or transactivation. EGFR transactivation is found in diverse cell types and various carcinoma cell lines (Schafer, et al., 2004) in response to a variety of stimuli (Rozengurt, 2007), (Thomas, et al., 2006).

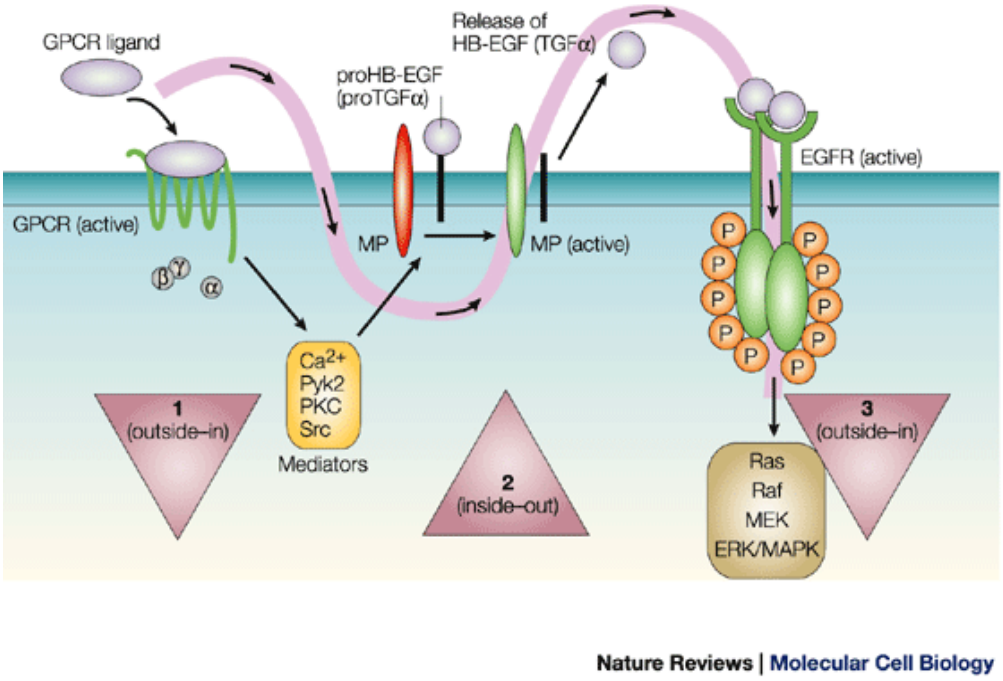


*Figure 12: EGFR transactivation via GPCR (Taken from (Wetzker; Bohmer, 2003)).*

The EGFR transactivation pathway involves three signalling steps traversing the membrane which has been designated triple-membrane-passing-signalling (Figure 12) (Daub, et al., 1996); (Chan, et al., 2006). Activation of GPCR accumulates in the modulation of secondary messengers such as Src-family kinases, calcium ($Ca^{2+}$), Pyk2 and protein kinase C (PKC), depending on the cell type (Figure 12). These secondary messenger cascades are involved in the activation of metalloproteases (MP, Figure 12) which are crucial elements for the paracrine or autocrine release of EGFR ligands. This process ensures that the signal passes the second time through the membrane (Figure 12, 2). The ligand binds its cognate receptor and activates the downstream cascade of this receptor (Figure 12, 3).

EGFR transactivation is deregulated by various cellular responses like overexpression, amplification or mutation of critical pathway elements with variable functional outcomes. EGFR transactivation is frequently linked to hyperproliferative diseases. GPCR induced EGFR transactivation mediates cell proliferation in breast cancer cells (Greco, et al., 2003), (Muscella, et al., 2003) and increases the tumourigenicity in ovarian cancer cells (Rosano, et al., 2007).

## 1.4 Protein kinases

A protein kinase is an enzyme that modifies other proteins by chemically adding phosphate groups (phosphorylation). The chemical activity of a kinase is described to transfer the phosphate molecule from ATP to an amino acid residue which has a free hydroxyl group. Mostly these kinases are defined on the basis of homologous catalytic domains on serine threonine

and tyrosine residues (app. 90%) (Hanks, et al., 1988), (Hanks; Hunter, 1995). Assessment of the phosphorylation is a major event on signal transduction mechanism used by eukaryotic cells to regulate proliferation, gene expression metabolism, motility, membrane transport and every cellular activity that defines their behaviour.

Protein kinases, encoded by approx. 2% of eukaryotic genes (Hunter, et al., 1997), represent the largest and most functionally diverse gene families. Here some of these kinases will be highlighted according to their functional activity and relation to ET-1.

### 1.4.1 Protein kinase A (PKA)

PKA is a cAMP-dependent enzyme (Smith, et al., 1993) which has been implicated in a wide range of cellular processes, including transcription (Huggenvik, et al., 1991), metabolism (Hubbard; Cohen, 1993), cell cycle progression (Matten, et al., 1994) and apoptosis (Gjertsen; Doskeland, 1995). Regulation of PKA in the cell is related primarily to the modulation of its phosphotransferase activity. The enzyme contains two regulatory and two catalytic subunits.

The PKA activation is modulated by factors which either activate or inhibit adenylate cyclase, resulting in an increase or decrease in cAMP levels. Activation proceeds by the cooperative binding of two molecules of cAMP to each regulatory subunit, which causes the dissociation of each active catalytic subunit from the regulatory subunit dimer.

### 1.4.2 AKTs (Protein kinase B)

AKTs, serine/threonine specific kinases, are also named Protein kinases B (PKB). The AKT family contains three genes AKT1, AKT2 and AKT3. AKT1 is involved in cellular survival pathways by inhibiting apoptotic processes. AKT2 is an important signaling molecule in the insulin signaling pathway. It is required to induce glucose transport. The role of AKT3 is less clear, though it appears to be predominantly expressed in the brain (Yang, et al., 2004).

AKT/PKB phosphorylation is regulated by the phosphorylation status of the lipid phosphatidylinositol (3,4,5)-triphosphate (PInsP3) (Shaw; Cantley, 2006). Activation takes place by multisite phosphorylation. The fully activated multiphosphorylated AKT then dissociates from the plasma membrane and targets substrates located in the cytoplasm and nucleus leading to the activation of genes involved in diverse cellular processes (Figure 13) (Hanada, et al., 2004).

AKT activation can be induced by a number of growth factors, including EGF (Gibson, et al., 1999), IGF-I (Alessi, et al., 1996) estrogen (Simoncini, et al., 2000), which all regulate mammary gland development (Coleman, et al., 1988) (Kleinberg, 1998), (Yang, et al., 1995),

(Fendrick, et al., 1998), (Richert, et al., 2000)). Fata and coworkers have implicated AKT activity in alveolar development during pregnancy (Fata, et al., 2000).

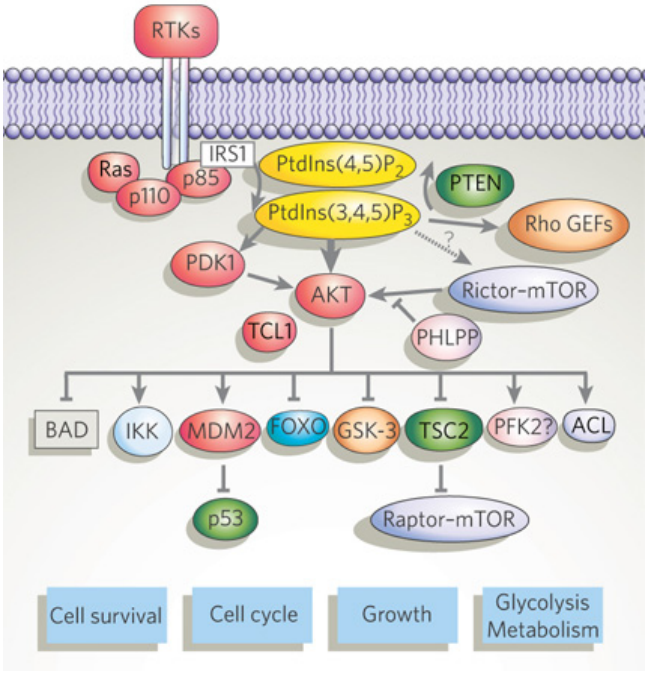


*Figure 13: AKT controls cell survival, cell cycle, cell growth and metabolism through phosphorylation of a number of key substrates (only some of which are shown in the Figure). Proteins whose genes are mutationally activated in human cancer are shown in red; those inactivated are in green (Taken from (Shaw; Cantley, 2006)).*

Te loss of AKT activation is correlated with impaired alveolar development in osteoprotegerin-ligand (OPGL) knockout mice. Substitution of OPGL by pellet implants in pregnant mammary glands results in an increase of phosphorylated AKT and the restoration of alveolar development. Constitutively active AKT in the mammary gland induces a delayed involution and a delay in the onset of apoptosis (Schwertfeger, et al., 2001) thus the reduction of AKT activity at weaning is necessary for a normal involution process.

All these reports suggest that AKT is involved in alveogenesis during pregnancy and lactation and regulation of normal mammary gland involution.

### 1.4.3 Protein kinase C (PKC)

PKCs are a group of serine/ threonine kinases that are divided into 3 subclasses (conventional, novel and atypical variants), based upon domain composition and cofactor requirements. All PKC isoenzymes contain a conserved kinase domain that is auto inhibited by the pseudo-substrate domain blocking access to the active site (Rozengurt, 2007).

The conventional isoenzyme group contains a C1 domain, a tandem repeat that binds either diacylglycerol or phorbol esters, and a C2 domain that binds the head group of phosphatidylserine in a calcium-dependent manner (Malbon, 2005), (Figure 9).

The presence of these domains in conventional PKC isoenzymes explains their requirements for DAG and calcium in activation of these PKC isoenzymes. The DAG activated group of isoenzymes contain a functional C1 domain but a non-functional C2-like domain, the C2 domain in these isoenzymes does not contain the amino acids required for calcium binding hence these isoenzymes are calcium-dependent. The atypical PKC isoenzymes do not contain a functional C1 or C2 domain (Pierce, et al., 2001), (Rozengurt, 2007). These monomeric enzymes are under tight structural and spatial regulation since the phosphorylation state, conformation and subcellular localisation must be precisely defined for physiological activity of the kinase. It is apparent that the individual PKC isoforms are differentially regulated by receptors, differentially localised in the cells and interact physically with a distinct set of signalling proteins. Functionally, the different isoforms are likely to play different roles in regulating activities in the cell (Malbon, 2005).

### 1.4.4 Extracellular signal regulated kinase (ERK)

ERKs or classical Mitogen Activated Protein Kinase (MAP) kinases are widely expressed intracellular signalling molecules which are involved in the regulation of meiosis, mitosis, and postmitotic processes in differentiated cells. ERKs are known to activate many transcription factors, and some downstream protein kinases.

The most well defined signalling pathway from the cell membrane to ERK 1 and ERK 2 is that used by EGFR (Hunter, et al., 1997), (Figure 14 A). ET-1 stimulates ERK 1 and ERK 2 through cognate receptors (GPCR) (Cramer, et al., 2001) via transactivation of the EGFR in various cell types and tissues (Hua, et al., 2003), (Kodama, et al., 2002). ET-1 elicits these responses predominantly through the activation of a $G\alpha_i$ linked (ETAR) cascade with a minor contribution from the $G\alpha_q$/PKC pathway. Selective inhibition of the EGFR causes inhibition of EGF induced ERK activation (Shah, et al., 2006). The ET-1 acts primarily via ETAR to induce phosphorylation of ERK1 and ERK2 in cultured vascular smooth muscle cells (Chen, et al., 2009) and induces proliferation (Zeidan, et al., 2003) (Figure 14 B).

Additionally, an unconventional signaling route that involves ERK1/2 phosphorylation controls cell death and tissue remodeling in the mouse mammary gland during involution which is partially induced by interleukin-6 (IL-6) and LIF (Zhao, et al., 2004).

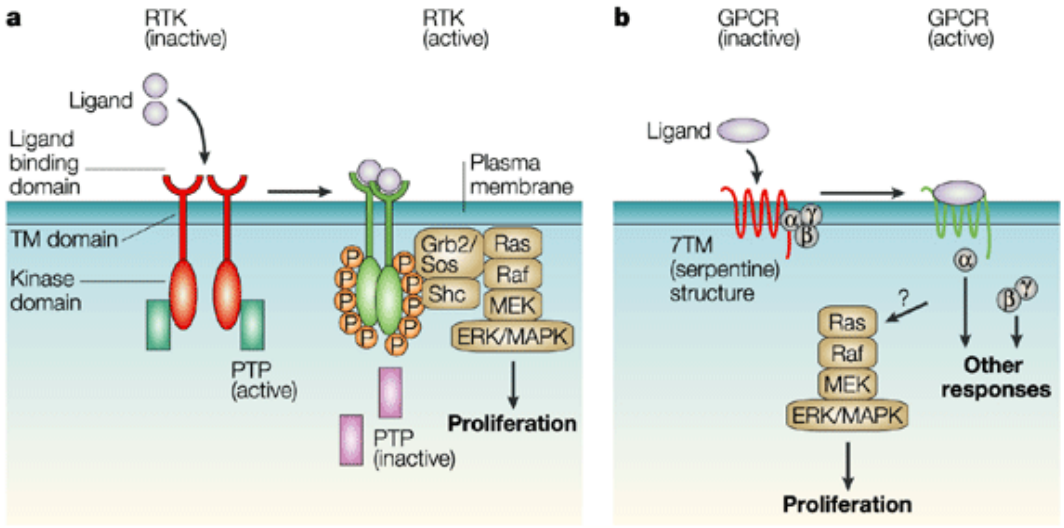


*Figure 14: Activation of ERKs, (a). After the activation of the EGFR, the adaptor molecules (Grb2, Shc) bind to the phosphotyrosine sites of the receptor which loads GTP onto Ras. Ras then activates the ERK/MAPK cascade through Raf and MEK. (b). Extracellular ligand binding to GPCRs leads to GTP-loading of the G∝ subunit and dissociation of the trimeric G-protein complex from the receptor. $G_{\propto}$ and $G^{\beta}\gamma$ subunits elicit intracellular signals through protein–protein interactions. Many GPCRs can activate the ERK/MAPK pathway and stimulate cell proliferation in some cell types (Taken from (Wetzker; Bohmer, 2003)).*

## 1.5 Signal transducer and activator of transcription (STAT) family

STAT proteins comprise a family of cytosolic transcription factors involved in a variety of cellular processes including mitogenesis (Yu; Jove, 2004), differentiation (Liu, et al., 1997) and apoptosis (Humphreys, et al., 2002) in response to many polypeptide cytokines and growth factors as downstream effectors (Schindler, et al., 1992), (David, et al., 1996). There are various signals to induce tyrosine phosphorylation of STATs (Figure 15). These pathways of activation are often referred to the JAK–STAT pathway. Upon activation of a cytokine, a growth factor receptor or a GPCR, the receptor initiates a cascade of tyrosine phosphorylation and activates receptor associated Jak (Janus protein kinase). This association ensures a docking place for STAT that recruits from the receptor a single phosphor on tyrosine residue. This phosphorylation triggers the release of STAT from the receptor and homo dimerization of activated STAT factor occurs very rapidly. Dimerized STATs translocate to the nucleus where STATs bind to the recognition site within the cognate promoter region (Chen, et al., 1998), (Neculai, et al., 2005)

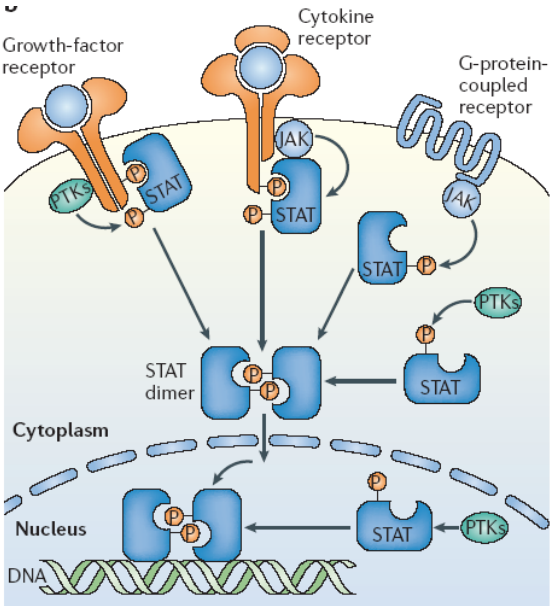


*Figure 15: Diagram of the multiple mechanisms for tyrosine phosphorylation of STATs by Janus kinases (JAKs) or other protein tyrosine kinases (PTKs) that are intrinsic to receptors or that are present in the cytoplasm or in the nucleus. Tyrosine phosphorylation generates STAT dimers that can bind specific DNA targets (Taken from (Reich; Liu, 2006)).*

There are seven mammalian STAT proteins that respond to distinct stimuli and induce the transcription of genes that can elicit different physiological outcomes. With the exception of STAT2, all STAT family members are expressed and developmentally regulated in the mammary tissue (Philp, et al., 1996). Analysis of the phosphorylation levels of STAT3 and STAT5 and their DNA binding activities exhibited a reciprocal relationship in mammary gland development (Philp, et al., 1996), (Liu, et al., 1997) (Figure 16).

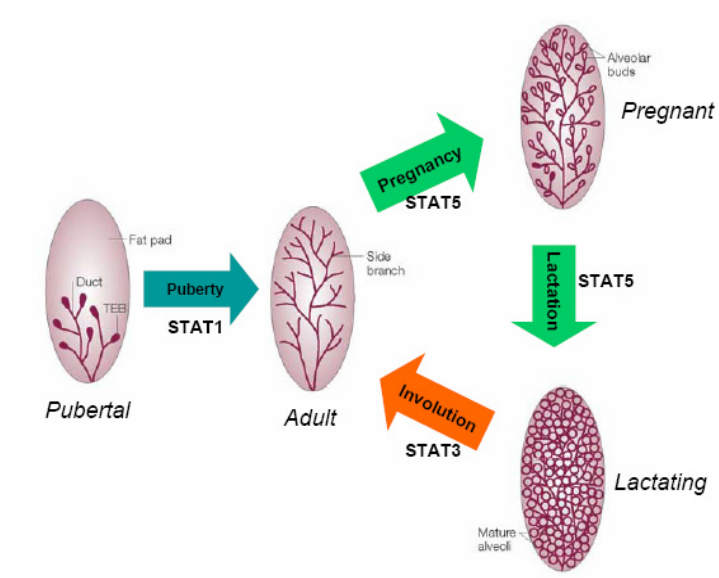


*Figure 16: The STAT family of transcription factors have diverse roles in mammary gland development. (According to (Watson; Neoh, 2008)).*

### 1.5.1 STAT 3

STAT3 was originally identified as an acute phase response factor, which is activated by IL-6 (Akira, et al., 1994), LIF (Kritikou, et al., 2003) and growth factors like EGF (Zhong, et al., 1994). In particular, in the normal mouse mammary gland the LIF-dependent activation of STAT3 is essential for apoptosis in mammary epithelium after weaning (Kritikou, et al., 2003). The natural activator of STAT3, IL-6 does not activate STAT3 for the initiation of involution in the mammary gland (Zhao, et al., 2004). In order to address the role of STAT3 in involution of the mammary gland, conditionally absence of STAT3 in a mouse model represented a 4 days delay of involution and caused a reduction of apoptosis (Chapman, et al., 1999). Since involution can be delayed, but not blocked, STAT3 appears necessary but not sufficient to induce mammary gland apoptosis (Baxter, et al., 2007).

Beside the critical function in the initiation of involution in the mammary gland, the biological activity of STAT3 depends on multiple factors. For example, an elevated STAT3 activation has been reported in breast cancer cell lines (Li; Shaw, 2002) and, the inhibition of STAT3 activity suppressed the proliferation and survival of these cancer cells in vitro (Garcia, et al., 2001), (Quaglino, et al., 2007).

#### 1.5.1.1 Leukemia inhibitory factor (LIF)

Expression of LIF is almost undetectable during the lactation period and increases after weaning to induce apoptosis in the mammary gland (Zhao, et al., 2004), (Schere-Levy, et al., 2003). Implantation of LIF containing pellets in lactating glands resulted in an induction of STAT3 phosphorylation and increased levels of apoptosis in these mammary glands (Schere-Levy, et al., 2003). This suggests that regulation of LIF expression is not depending on circulating lactational hormones.

LIF is involved in the pathogenesis of breast cancer by the finding of LIF expression in MDA-MB 231 breast cancer cells (Estrov, et al., 1995). LIF, as a multi functional cytokine was found to stimulate proliferation of several breast carcinoma cells in vitro (Bamberger, et al., 1998).

### 1.5.2 STAT 5

The mammary gland undergoes dramatic morphological changes during pregnancy, lactation, and involution. These defined stages of postnatal mammary gland growth and differentiation are controlled by the synergistic action of hormones and local growth factors as well as their downstream effectors. The expression and activity of STAT5 is closely linked to the onset of lactation, whereas the expression and activity of STAT1 and STAT3 is associated with the virgin mammary gland and involution (Watson, 2001).

STAT5 is activated by prolactin (Jaroenporn, et al., 2009) but also by growth hormones and the EGFR (Gallego, et al., 2001). Hereby, the STAT5 is critical for the growth and differentiation of alveolar progenitors as well as the survival of secretory mammary epithelial cells. Activated STAT5 is a critical regulator for the milk protein gene expression of Beta-casein and WAP (Li; Rosen, 1995), (Happ; Groner, 1993). Therefore the STAT5 activity has been considered as a marker for secretory differentiation and alveolar expansion. It has been noted that depletion of STAT5 in a knockout model exhibited multiple abnormalities in a variety of organ systems including female infertility and impaired mammary gland development (Teglund, et al., 1998).

## 1.6 Aim of the study

Transgenic mice generated by DNA microinjection represent a powerful tool to address basic questions on gene regulation and gene function within an intact organism. Furthermore, they represent an attractive in vivo model to induce disease processes and to analyze the underlying mechanisms in order to understand at the molecular level the contribution of a given gene to the entire disease cascade. Based on these assumptions Theuring and coworkers have generated transgenic mice expressing the human ET-1 gene to study the role and function of this potent vasoactive molecule in cardiovascular physiology (Hocher, et al., 1997).

As described previously, ET-1 was not only found to play an important role as vasoactive substance, but also contributes prominently to various developmental processes. In support of this, ET-1 transgenic female mice delivering newborns had been found in this study to be unable to nourish their pubs appropriately with milk and this interesting observation provided the basis for this experimental thesis work. Consequently, the aim of this study was to investigate in more detail the influence of ET-1 expression on the different developmental stages of the mouse mammary gland during pregnancy, lactation and involution by employing a comparative histological, immunohistochemical, biochemical and molecular analysis. In parallel, some of the relevant questions were also addressed by using mammary epithelial cell specific cell lines in vitro.

These combined analyses should provide a better understanding of the underlying mechanisms leading to the observed defects such as secretory activation, precocious involution and lactational hyperplasia in the mammary glands of human ET-1 transgenic female mice.

# 2 Materials and Methods

## 2.1 Materials

### 2.1.1 Laboratory chemicals and biochemicals

| | |
|---|---|
| Acrylamide/bisacrylamide (30:1) | Roth |
| Agarose | Biozym |
| Aluminium potassium sulphate | Sigma |
| Ammonium Persulphat | Gibco BRL |
| BSA | Applichem |
| Carmine dye | Sigma |
| Chlorophorm | J.T.Baker |
| DEPC | Sigma |
| DMSO | Sigma |
| dNTPs | Biomol |
| EDTA | Sigma |
| Entellan | Merck |
| Eosin | Merck |
| Ethanol (100%) (MEK) | Herbeta Arzneimittel |
| Ethanol (100%) (Molecular grade) | J.T.Baker |
| Ethidium bromide | Serva |
| Ficoll-400 | Sigma Aldrich |
| Formalin solution | Sigma Aldrich |
| Glycine | Roth |
| $H_2O_2$ | Sigma |
| Haematoxylin | Merck |
| HCl | Merck |
| Isoprophanol | J.T.Baker |
| Methanol | J.T.Baker |
| MTT | Sigma |
| $NA_3VO_4$ | Sigma |
| NaCl | Merck |
| NaF | Roth |
| $NaN_3$ | Sigma |
| NaOH | Merck |
| Phosphatase inhibitor cocktail (Tablet) | Roche |

| | |
|---|---|
| Ponceau S | Sigma |
| Protease inhibitor cocktail (Tablet) | Roche |
| Rna ZAP | Ambion |
| SDS | Serva |
| Sodiumdeoxycholate | Sigma |
| TEMED | Biorad |
| Tris HCl | Roth |
| Trizol | Invitrogen |
| Tween20 | Merck |
| Xylol | J.T.Baker |

All other chemicals were purchased from Sigma Aldrich.

### 2.1.2 Ligands, agonists, antagonists and enzymes

| | | |
|---|---|---|
| 0.5%Trypsin-EDTA | | Gibco |
| Taq DNA Polymerase | | Invitek |
| Taq DNA Polymerase | | Eppendorf |
| Syber Green PCR Master mix | | Applied Bio |
| Active EGF | | Biomol |
| Active human porcine Endothelin-1 | | Sigma |
| Forskolin | | Fluka |
| Selective ETAR blocker | BQ 123 | Sigma |
| Selective ETBR blocker | BQ 788 | Sigma |
| Reverse Transcriptase | M-MLV | Promega |
| IBMX | | Sigma |
| BMI | | Sigma |
| RNAsin | | Promega |
| DNAse | | Ambion |

### 2.1.3 Standards and markers

| | |
|---|---|
| 1 kb DNA Ladder | Fermentas |
| 100 bp DNA Ladder | Fermentas |
| BSA protein standard | Perbio (2 mg/ml stock) |
| Prestained protein Ladder | Fermentas (10 to 170 kDa broad range) |

### 2.1.4 Kits and other materials

| | | |
|---|---|---|
| 4x protein assay reagent | Bradford | Biorad |
| 4x protein loading buffer | GLD | Roth |
| BrDU proliferation assay kit | | Roche |

| | | |
|---|---|---|
| cAMP assay kit | cAMP-Glo | Promega |
| Chemiluminescence Film | Biomax | Kodak |
| ECL Kit | | Amersham |
| Filters | 22 µm | Whatman |
| IHC ABC kit | vectastain elite | Vector laboratories |
| Laboratory film | Parafilm | Roth |
| Microtome blades | A35 | Feather |
| Neubauer chamber | | Menzel Glaser |
| Nitrocellulose Membrane | Hybond-ECL | Amersham |
| PCR plates | 96 well | Sarstedt |
| PCR Tubes | 8 well | Biozym |
| Plate sealing tape | | Sarstedt |
| Protein G Sepharose | 4 Fast Flow | GE Healthcare |
| RNA analyse kit | RNA 6000 nano kit | Agilent Tech |
| RNAase inhibitor | Turbo Rnase | Ambion |
| RNAzap | | Ambion |
| Slide | Superfrost plus | R.Langenbrinck |

All disposable cell culture materials, flasks, scrapers and plates were purchased from Sarstedt, falcon tubes and pipettes were purchased from BD.

### 2.1.5 Cell culture mediums and buffers

All culture media and additives were purchased from Gibco and isotonic water from Pan tech. Dulbecco`s modified eagle medium (DMEM) supplemented with 4,5 mg/ml L D Glucose, 25mM HEPES, without pyruvate was employed. The medium was supplemented with 10%FCS and 1% penicillin-streptomycin.

1x PBS conditioned as pH 7,2, -$CaCl_2$, -$MgCl_2$

The cell culture freezing medium contained 90% FCS, 10% DMSO

### 2.1.6 Synthetic oligonucleotide PCR primers

Ensembl gene data base and commercial software (Primer3) and web based algorithms (NCBI Blast, multi align) were used in the design of oligoprimers which were not referenced. All primers contained a splice boundary within a message sequence so that no genomic DNA was amplified. All these Primers were purchased from Invitrogen, Oligo $(dT)_{15}$ and random primers were obtained from Promega. The primers sequence is listed in Table 2.

*Table 2: Primer sets for PCR applications*

| Origin | Oligonucleotide | pr. | Sequence 5'-->3' | Product Size (bp) | TM °C | Genbank accesion no. |
|---|---|---|---|---|---|---|
| *mouse* | **Alpha-** | *F* | CTCCTGTGACAAGTTATTGGATGAC | 100 | 62 | ENSMUST00000023726 |
| | **lactalbumin** | *R* | CTTGTAGGCTTTCCAGTAGTCGATTC | | | |
| *mouse* | **Amphiregulin** | *F* | TCTTGGGCTTAATCACCTGTTC | 100 | 60 | NM_009704 |
| | | *R* | GGGGACTACGACTACTCAGAG | | | |
| *human* | **Amphiregulin** | *F* | CCCAAAACAAGACGGAAAGTGA | 189 | 61 | NM_001657 |
| | | *R* | GCTGACATTTGCATGTTACTGCT | | | |
| *mouse* | **Beta- Casein** | *F* | CTCCACTAAAGGACTTGACAGC | 213 | 59 | ENSMUST00000082370 |
| | | *R* | AGTGAACTTTAGCCTGGAGCAC | | | |
| *mouse* | **EGF** | *F* | CTAAGGATCCTGACCCCGAACT | 175 | 60 | . |
| | | *R* | GTACAGCCGTGATTCTGAGTGG | | | |
| *mouse* | **EGFR** | *F* | GCCAATAATGTCTGCCACCT | 118 | 60 | . |
| | | *R* | TCCCAGTGGCAATAGATGGT | | | |
| *mouse* | **ETAR** | *F* | GCTGGTTCCCTCTTCACTTAAGC | 129 | 60 | BC008277 |
| | | *R* | TCATGGTTGCCAGGTTAATGC | | | |
| *human* | **ETAR** | *F* | CTTAGTGTTGACAGGTACAGAGCAGT | 209 | 60 | ENST00000324300 |
| | | *R* | ATGAATTTTGATGTGGCATTGA | | | |
| *mouse* | **ETBR** | *F* | TGTGCTCTAAGTATTGACAGATATCGAG | 240 | 60 | U32329, |
| | | *R* | GGCTGTCTTGTAAAACTGCATGA | | | |
| *human* | **ETBR** | *F* | GAGTATTGACAGATATCGAGCTGTTG | 181 | 60 | ENST00000334286 |
| | | *R* | GCAGATTCGCAGATAACTTCCT | | | |
| *mouse* | **GAPDH** | *F* | CTTCACCACCATGGAGAAGGC | 238 | 60 | M32599;M17851 |
| | | *R* | GGCATGGACTGTGGTCATGAG | | | |
| *mouse* | **GLUT-1** | *F* | CCAGCTGGGAATCGTCGTT | | 60 | |
| | | *R* | CAAGTCTGCATTGCCCATGAT | | | |
| *mouse* | **HB-EGF** | *F* | TGAACCTTTTCAAAGTTGCTTTCT | 150 | 60 | |
| | | *R* | CGTGGATGCAGTAGTCCTTGTA | | | |
| *mouse* | **IL6** | *F* | TGTACTCCAGGTAGCTATGG | | 60 | . |
| | | *R* | GTTCTCTGGGAAATCGTGGA | | | . |
| *human* | **IL6** | *F* | ACCCCCAGGAGAAGATTCCA | | 60 | |
| | | *R* | CCCAGTGGACAGGTTTCTGA | | | |
| *mouse* | **LIF** | *F* | GGCAACCTCATGAACCAGATCA | 335 | 59 | . |
| | | *R* | GCAAAGCACATTGCTGAGGAGGC | | | |
| *human* | **LIF** | *F* | GGCCCGGACACCCATAGACG | 454 | 59 | . |
| | | *R* | CCACGCGCCATCCAGGTAAA | | | |
| *mouse* | **PPET-1** | *F* | CCCCACTCTTCTGACCCCTT | 213 | 60 | ENSMUST00000021796 |
| | | *R* | ACTCCTTGTCCATCAAGGAAGAAC | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *human* | **PPET-1** | *F* | ATCAGAAGAAGTTCAGAGGAACAC | 153 | 62 | ENST00000379375 |
| | | *R* | GAAGGTCTGTCACCAATGTGCT | | | |
| *human* | **PPET-1** | *F* | CCCCATTCTAAGCATAGGGGC | 503 | 60 | |
| | ***genomic*** | *R* | AGCCAGTGAAGATGGTTGGGG | | | |
| *H&M* | **PPET-1** | *F* | CAAGGAGCTCCAGAAACAGC | 168 | 58 | ENSMUST00000021796 |
| | | *R* | GATGTCCAGGTGGCAGAAGTA | | | ENST00000379375 |
| *mouse* | **SRBF-1** | *F* | AGGCGGCTCTGGAACAGA | | 60 | |
| | | *R* | TGTCGTTCAAAACCGCTGTG | | | |
| *mouse* | **TGF-a** | *F* | CAGAGGGCAGTACAGTTGATTCAG | 193 | 60 | |
| | | *R* | GAAGACATCCTGGGCAAGC | | | |
| *mouse* | **WAP** | *F* | TAGCAGCAGATTGAAAGCATTATG | 500 | 60 | . |
| | | *R* | CAACGCATGGTACCGGTGTCA | | | |

### 2.1.7 Antibodies

The following primary antibodies were used in immunoprecipitation experiments and Western blot analyses (Table 3).

*Table 3: List of primary antibodies with their origins and working dilutions.*

| Antibody | Origins and Dilutions | Molecular weight (kDa) | Reference |
|---|---|---|---|
| pAKT | Rabbit polyclonal antibody 1/1000 dilution | 60 | Santa Cruz Biotech. |
| AKT | Rabbit polyclonal antibody 1/1000 dilution | 60 | Santa Cruz Biotech. |
| B-Actin | Goat poyclonal antibody 1/2000 dilituon | 43 | Santa Cruz Biotech. |
| pEGFR (tyr 845) | Rabbit polyclonal antibody 1/1000 dilution | 170 | Cell signaling Tech. |
| EGFR | Rabbit polyclonal antibody 1/1000 dilution | 170 | Santa Cruz Biotech. |
| pERK | Rabbit polyclonal antibody 1/1000 dilution | 42/44 | Cell signaling Tech. |
| ERK | Rabbit polyclonal antibody 1/1000 dilution | 42/44 | Cell signaling Tech. |
| ETAR | Rabbit polyclonal antibody 1/2000 dilituon | 69 | Santa Cruz Biotech. |
| ETBR | Rabbit polyclonal antibody 1/2000 dilituon | 49/34 | Santa Cruz Biotech. |

| | | | |
|---|---|---|---|
| SMA | Mouse monoclonal antibody 1/4000 dilituon | -- | Sigma |
| pSTAT3 (tyr 705) | Rabbit polyclonal antibody 1/1000 dilution | 79/86 | Cell signaling Tech. |
| STAT3 | Rabbit polyclonal antibody 1/1000 dilution | 79/86 | Cell signaling Tech. |
| pSTAT5 (tyr 694) | Rabbit polyclonal antibody 1/1000 dilution | 90 | Cell signaling Tech. |
| STAT5 | Rabbit polyclonal antibody 1/1000 dilution | 90 | Cell signaling Tech. |

The following secondary antibodies are conjugated with horseradish peroxydase (HRP) and utilized for Western blot analyses (Table 4).

*Table 4: List of secondary antibodies with their origins and working dilutions.*

| Antibody | Origins and Dilutions | Reference |
|---|---|---|
| Antimouse Ig | Rabbit, HRP linked polyclonal antibody 1/2000 | Dakocytomation. |
| Antirabbit Ig | Goat, HRP linked polyclonal antibody 1/2000 | Dakocytomation. |
| Antigoat Ig | Rabbit, HRP linked polyclonal antibody 1/2000 | Dakocytomation. |

### 2.1.8 Cell lines

Cell lines have been utilized in line with the recommendation of the American Type Culture Collection (ATCC).

MCF7 (ATCC number: HTB-22) Human breast adenocarcinoma cells

MDA MB 231 (ATCC number: HTB-26) Human malignant breast adenocarcinoma cells

### 2.1.9 Animal models

For this study, mice of the out bred strain NMRI (Harlan-Winkelmann, Paderborn) as wild type and NMRI originated homozygotic human ET-1 transgenic mice (generated by Prof.Dr Franz Theuring) were used. Mice were kept in a pathogen free animal facility with controlled light and dark cycle (12 h light, 12 h darkness) and temperature (20-22 °C) and relative humidity (50-70%). Animals had free access to standard mouse chow (mouse chow 5015) and tap water. The mice were routinely screened for common mouse pathogens.

### 2.1.10 Stock solutions and buffers

The following standard buffers and media were modified from “Molecular Cloning” (Sambrook, 1989) for basic molecular biology applications. Without further notification, $H_2O$ used was deioinised and ultrafiltered using a millipore apparatus. The pH was adjusted with HCl or NaOH, respectively.

- **Agarose (1%):**

  1% Agarose was boiled in 1x TAE buffer with a microwave oven until the agarose dissolved. The solution slowly cooled down to ~35 °C and then 6% ethidium bromide was added and stored at 58 °C in a water bath.

- **APS (10%):**
  10 g APS was dissolved and adjusted to 100 ml for aliquoted to 1 ml for each vial and stored at -20 °C

- **Carmin allum dye:**
  2% Carmine dye (wt/Vol), 5% aluminium potassium sulphate (wt/Vol) was boiled for 20 min, filtered and stored at 4 °C

- **Carnoy`s Fixative:**
  75% Ethanol, 25% Glacial Acetic Acid were mixed and stored at room temperature.

- **DEPC water:**
  1ml DEP was adjusted to 1000 ml.

- **Destainer for carmin:**
  350 ml EtOH (absolute alcohol) and 28,2 ml HCl (36-38 %) were mixed and adjusted to 500 ml.

- **Dilution buffer:**
  4% BSA, 0.1% Tween20 dissolved in TBS.

- **DNA/RNA loading buffer (10X)**
  100 mM Tris HCl (pH: 7.4), 0.5 M EDTA, 25% Ficoll-400, was added to a final volume of 10 ml with $ddH_2O$, heated to 65°C to dissolve. Finally 25-50 mg of each, xylene cyanol and Orange G was added.

- **Electrophoresis buffer (5x):**
  0,125 mM Tris Base (pH: 8.3), 1.25 M Glycine, 10% SDS stored at room temperature as a stock solution.

- **RIPA buffer:**
  50 mM Tris HCl (pH: 7.5),150 mM NaCl, 50 mM NaF, 200 µM $NA_3VO_4$ (activated), 0,5% sodium deoxycholate, 0,1% SDS, 0,02% $NaN_3$. RIPA buffer was supplemented with a protease inhibitor cocktail tablet and a phosphatase inhibitor cocktail tablet before use.

Sodium orthovanadate should be activated for maximal inhibition of protein phosphotyrosyl phosphatase. The activation of sodium orthovanadate was performed according to the protocol of (Gordon, 1991). The supplemented buffer was stored at 4 °C for 2 weeks.

- **SDS (10%):**
  10 g SDS was dissolved and adjusted to 100 ml and stored in darkness at room temperature.
- **Separation gel buffer:**
  1.5 M Tris HCl (pH: 8.8), 0.4 % SDS.
- **Stacking gel buffer:**
  0.5 M Tris HCl (pH: 6.8), 0.4 % SDS.
- **Stripping buffer:**
  0124 mM Glycine (pH: 2), 10%SDS stored at room temperature.
- **TAE buffer (10x):**
  400 mM Tris Acetate (pH: 8.0), 10 mM EDTA stored at room temperature.
- **TBS (10 x):**
  200mM Tris Base (pH : 7.6), 1,37 M NaCl
  The buffer was stored at room temperature.
  TBS-$T_{20}$ =10% 10x TBS, 1% Tween 20, 89% $dH_2O$. The TBS-$T_{20}$ buffer was used as immunoblott washing solution and immunoblott membrane blocking solution with the addition of 4% BSA.
  The blocking solution was prepared fresh before use and was also utilized as an antibody dilution solution.
- **TE buffer (10 x):**
  10mM Tris HCl (pH: 8.0), 5 mM EDTA stored at room temperature.
- **Tissue washing buffer :**
  50mM Tris (pH: ~7), 150 mM NaCl stored at 4 °C.
- **Transfer buffer (4x):**
  120 mM Tris Base, 92 mM Glycine stored at room temperature as stock solution.
  Transfer buffer= 20% 4x Transfer buffer, 20% Methanol, 60% $dH_2O$ (pH~8,3 without adjustment), stored at 4 °C.
- **Urea buffer:**
  Buffer A: 25 mM Tris HCl, 50mM KCl, 3mM EDTA, stored at -20°C
  Buffer B: 7 M Urea, 2 M Thiourea, 70 mM DTT, fresh
  Prior to protein isolation Buffer B was mixed and dissolved in buffer A (urea buffer) after

that 1 tablet of protease and 1 tablet of phosphatase inhibitor cocktail (Roche) was added to the Urea buffer.

## 2.2 Methods

### 2.2.1 Animal handling and experiments

The animals were sacrificed by cervical dislocation. In general male and female mice were held separately in groups of 2-6 animals. All animal experiments were conducted in accordance with the German Law for Animal Protection (Tierschutzgesetz).

Mammary gland samples were obtained free of the muscles of the anterior abdominal wall and skin by blunt dissection. The fat cushion containing the 3rd, $4^{th}$, $8^{th}$ and $9^{th}$ positioned mammary gland was dissected and rinsed in ice cold tissue washing buffer. Afterwards probes were quickly marked, separated and placed in $LN_2$ for molecular studies or placed in formalin solution (Sigma) for histopathology studies or spread out on a glass slide for whole mount staining, respectively. The number of animals per experimental group is listed in Table 5.

*Table 5: Experimental groups and number of mice*

| | Virgin | | Pregnancy | | Lactation | | Involution |
|---|---|---|---|---|---|---|---|
| | *3 weeks* | *8 weeks* | *10th day* | *18th day* | *3 th day* | *14th day* | *14th day* |
| WT | 5 | 6 | 6 | 7 | 6 | 10 | 7 |
| ET-1 | 5 | 6 | 6 | 7 | 6 | 10 | 7 |

The weights of newborn animals were measured at the given days throughout the lactation period: new born, 3, 7, 11, 14, 17, 21st days of lactation. The number of pups nursing on wild type (n=10) dams were restricted to the same as that nursing on ET-1 transgenic dams (n=17). Each mother was allowed to nurse only 7 pups.

### 2.2.2 Isolation of genomic DNA from tissue

At the end of the lactation period, 3 weeks old pups were separated from their mother and the tip of the tail from each mouse was excised. For genomic DNA isolation, the tip of the tail was incubated in 700µl of lysis buffer1 containing 35 µl proteinase K (10µg/µl) at 55 °C for ~2 h in a thermo shaker.

An equal volume of isopropanol was added to the lysate, and after vortexing the mixture was centrifuged at 8000x g at room temperature for 10 min. The pellet was washed 3 times with 1 ml 70% ethanol and dried at room temperature and dissolved in 200µl of TE buffer.

### 2.2.3 Paraffin embedding of mouse mammary glands

For histological analysis the mammary glands were fixed in 4% (w/v) Paraformaldehyde in PBS, (pH 7.4) for a minimum of 12 hours at room temperature. After fixation mammary glands were placed in a tissue cassette and washed with PBS.

The embedding was performed with a tissue processor, Shandon Citadel 1000 (Thermo) and a fully automated program was run with the following protocol:

| | |
|---|---|
| -70% ethanol | 3 hours |
| -80% ethanol | 1 hour |
| -95% ethanol | 1 hour |
| -100% ethanol 1 | 1 hour |
| -100% ethanol 2 | 1 hour |
| -xylol 1 | 1 hour |
| -xylol 2 | 2 hours |
| -paraffin 1 | 2 hours |
| -paraffin 2 | over night |

Parafin steps were performed at 60°C. The paraffin-embedded mammary glands were sectioned at 5µm thickness with a microtome HM 325 (Microm). Sections were stained with Hematoxylin-Eosin Mayer staining.

### 2.2.4 Hematoxylin –Eosin (Mayer) staining

- Sections were dewaxed in xylene, two changes and 10 minutes each.
- Sections were then rehydrated by passing through graded alcohol (two changes, absolute ethanol, 30 seconds each, followed by 96% ethanol, 80% ethanol, 70% ethanol, 30 seconds each and rinsed in water afterwards.
- Sections were incubated for 5 minutes in Ehrlich's Hematoxilin and blued for 5 minutes in tap water.
- Sections were dehydrated by passing through graded alcohol and mounted in entellan.

### 2.2.5 Whole mount staining

The mammary gland whole mounts were prepared using the axillar (3rd) mammary gland. Glands were removed and spread on a microscope slide. After 5 min air drying, the microscope slide was placed in Carnoy´s fixative overnight at room temperature. The epithelium was stained in alum carmine overnight. Next, the slides were destained for 2 hr to remove excessive dye from the gland. Following destaining, the mammary glands were dehydrated by incubating in four sequential steps of alcohol (35–95%, for 30 min each and 100% alcohol

overnight). Glands were defatted and cleared in xylene for 24 h and stored in xylene (Rowland 2002).

The structure of the mammary glands was analyzed under a stereo microscope and documented with a digital camera (Canon). Whole mounts were evaluated for TEB, alveolar lobe, and lobular structures according to the criteria of Russo (Russo, et al., 1990).

### 2.2.6 Immunohistochemical staining

5 mm thick sectioned paraffin slides were deparafinized with xylene and dehydrated through increasing series of alcohol grades and washed with dd$H_2O$. In order to remove endogenous peroxidase activity sections were incubated in 0,3% $H_2O_2$ for 30 min. After washing, sections were incubated with diluted blocking serum for 20 min which was prepared from species in which the secondary antibody is made.

Afterwards, the slides were incubated for 30 min with smooth muscle actin (1/4000 diluted in dilution buffer), washed for 5 min and incubated for 30 min with 1/1000 diluted biotinylated antimouse secondary antibody. After this point the protocol was performed according to manufacturer's (Vector, vectastain ABC Elite) recommendations.

### 2.2.7 Histopathological analysis

A total of 232 mammary gland samples (2 slides from each mammary gland and 4 mammary glands from each mouse) was analyzed from pregnant (n=26) and lactating (n= 32) females and stained with Hematoxylin –Eosin. These slides were histopathologically evaluated with a blinded analytical protocol.

A microscope (Zeiss, Axiovision imager M1) was used for histological analyses and photographic documentation. In addition, for the measurements of alveolar size of the mammary glands and post photographical work, the Axiovision software (Zeiss) was employed.

### 2.2.8 General cell culture techniques

Cells were checked every day macroscopically and microscopically and the cell density on the flask bottom or plate bottom was observed. When the cells reached a 90 % confluence, the old medium was removed and washed with pre-warmed (37°C) PBS.

After rinsing cells with 1 ml of PBS (enough amount to cover the surface of the flask bottom) sterile trypsin-EDTA was added and incubated 1-2 min to remove adherent cells from the flask. Addition of 6 ml 10% FCS supplemented to DMEM medium was used to neutralize the trypsin´s action. After this point cells were splitted on new flasks or seeded into 96 and 6 well plates to continue cell culture experiments or frozen to keep cells for a long time (Freezing medium contained 90%FCS and 10% DMSO). All cell lines were cultured in an incubator

(Heraus, Heracell 150) with humidified air (95%) and $CO_2$ (5%) at 37°C and before seeding cells were counted with a Neubauer chamber.

#### 2.2.8.1 In vitro experiments

Either 4000 cells /ml of the MCF7 cell line or 2000 cells /ml of the MDA MB 231 cell line in 10 % FCS supplemented medium were seeded in 96 well plate to perform viability experiments. Gene expression and protein analysis were carried out in 6 well plates and about 400000 cells were seeded into each well.

In a 24hr period, cell cultures reached to a ~80% confluency for each well. Prior to the assay application, the medium was removed, cells were washed with PBS medium and then the medium was refreshed with DMEM (without FCS) and cells were cultured again for 24 hr. After 24 hr of incubation the medium was refreshed with DMEM (without FCS) medium which contained bioactive molecules (Table 6). In order to analyse the protein phosphorylation, cells were treated for 10 min and for RNA expression analyses cells were treated for 1hr with various combinations of bioactive molecules.

*Table 6: Bioactive molecules and their application concentrations*

| | **Solvent** | **Stock solution** | **Application concentration** | **Stored at** |
|---|---|---|---|---|
| BQ788 | DMEM+(P/S) | 100mM | 100µM | –20 $^0$C |
| BQ123 | DMEM+(P/S) | 100mM | 100µM | –20 $^0$C |
| Porcine ET-1 | DMEM+(P/S) | 200µM | 10µM | As powder –20 $^0$C |
| IBMX | DMSO | 100 mM | 500µM | –20 $^0$C |
| EGF | DMEM+(P/S) | 100 mM | 10nM | –20 $^0$C |
| Forskolin | DMSO | 100mM | 100µM | –20 $^0$C |

### 2.2.9 Biochemical and cell biological assays

#### 2.2.9.1 MTT proliferation assay

The MTT assay is a colorimetric bioassay which is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave Yellow MTT which is a tetrazole, to the reduced purple formazan, which is largely impermeable to cell membranes. The number of surviving cells is directly proportional to the level of the formazan product created. Solubilisation of the cells by the addition of DMSO results in the dissolution of the purple formazan crystals. The absorbance of this colored solution was quantified by an Elisa reader (Biorad)

measuring at a 500 nm wavelength. The method was conducted as described previously (Mossman, 1983).

#### 2.2.9.2 BrDU incorporation proliferation assay

The BrDU assay is a nonradioactive alternative to the [3H]-thymidine-based cell proliferation assay. The assay is based on the measurement of cell proliferation by quantitating BrDU incorporated into the newly synthesized DNA of replicating cells. The assay is a cellular immunoassay which uses a mouse monoclonal antibody directed against BrDU. The BrDU assay was performed according to the manufacturer´s (Roche) recommendations.

#### 2.2.9.3 cAMP assay

This method was performed in parallel with studies of active ET-1 induced cell stimulation to demonstrate ET-1 induced receptor activations. The cAMP-Glo™ Assay is a homogeneous, bioluminescent and high-throughput assay for measuring cAMP levels in cells. The cAMP-Glo™ Assay monitors cAMP production in cells in response to the effects of test compounds on GPCR. GPCRs that couple with adenylate cyclase will increase or decrease intracellular cAMP. The assay is based on the principle that cyclic AMP (cAMP) stimulates protein kinase A (PKA) holoenzyme activity, thereby decreasing available ATP and leading to decreased light production in a coupled luciferase reaction. The assay was performed according to the manufacturer (Promega) recommendations.

### 2.2.10 Protein analytical methods

#### 2.2.10.1 Total protein isolation from mammary glands

Frozen mammary gland samples were pulverized in $LN_2$ with a mortar and a pestle. Homogenized tissue was aliquoted for RNA and protein based studies. 100mg tissue samples were mixed with 600 µl Urea buffer. Vigorously shaken samples were incubated for 15 min at room temperature. After solubilisation the samples were centrifuged at 13000 rpm for 45 min at room temperature. The supernatants were placed into new tubes.

#### 2.2.10.2 Total protein isolation from cell culture

After the incubation, medium was discarded and cells were washed with ice cold isotonic water. Isotonic water was discarded and ice cold 100 µl RIPA buffer was placed into each well. With a sterile scraper adhesive cells were harvested and homogenized in the buffer. Homogenates were placed into eppendorf tubes. Samples were incubated 5 minutes for protein solubilisation on ice and samples were centrifuged at 13000 rpm for 45 min at room temperature. The supernatants were placed into new tubes and any debris was discarded.

#### 2.2.10.3 Determination of protein concentration

To determine the protein concentration, a Bradford assay (Biorad) was used. 2 mg/ml BSA solution (Perbio) was diluted in order to obtain standard dilutions in the range of 25µg/ml to 200µg/ml. The 4x Bradford reagent was diluted to 1x Bradford reagent and 100 µl 1 x reagent was pipetted into each well of a flat bottom 96 well plate.

All unknown concentration samples were diluted to 1:25. Then the samples standard dilutions and blanks were pipetted (10µl) on to Bradford reagent; all samples and standards were done in triplicate. The plate was incubated in the darkness for 15 min at room temperature. Finally the absorption of the colorimetric reaction was measured at 595 nm with an ELISA reader (Benchmark Biorad).

#### 2.2.10.4 Immunoprecipitation

Grinded mammary gland samples were lysed in ice cold RIPA buffer. Lysates containing 400 µg of proteins in RIPA buffer were incubated with 2µg/ml EGFR antibody at 4°C overnight. The immune complex was precipitated with Protein A Sepharose CL-4B beads at 4°C for 1hr. The beads were washed (3 times) with PBS containing tween-20 (1%) and spin down by centrifugation (2000 rpm for 1 min), re-suspended in RIPA buffer and boiled for 5 min at 95 °C in a thermomixer (Eppendorf). The samples were spin down at 2000 rpm for 1 min. The supernatants were carefully placed in new tubes and immunobloted with EGFR pY845 and EGFR y1005, respectively.

#### 2.2.10.5 SDS-PAGE

SDS gel electrophoresis is a method for separating proteins according to their size from a mixed protein sample. The proteins were denaturized by boiling at 95 °C for 5 min in the presence of reducing agents (4x loading buffer, Roth). Meanwhile, all the proteins were coated by SDS which is a negatively charged detergent which ensures the separation of proteins by their molecular weights (Mw). Different gel concentrations were employed according to the different Mws of the interested proteins (see Table 7) to enhance the resolution of the protein bands.

*Table 7: Molecular weights of analyzed proteins and optimized separation gel concentrations for each protein.*

| Protein | Mw (kDa) | Gel concentration (%) |
|---|---|---|
| pEGFR, EGFR | 170 | 6 |
| pSTAT5, STAT5 | 90 | 10 |
| pSTAT3, STAT3 | 79/87 | 10 |
| ETAR | 69 | 10 |
| p AKT, AKT | 60 | 12 |
| pERK, ERK | 42/44 | 12 |
| ETBR | 49/34 | 12 |
| Beta-actin | 43 | 12 |

A SDS PAGE gel consists of two gels; first a separation gel (for the different concentration see Table 8) was poured into the electrophoresis cassette (Biorad mini protean II). In order to achieve a smooth boundary between separating and stacking gel, the separation gel was covered with a layer of 2-propanol. After the polymerisation of the separation gel was completed, the stacking gel (Table 9) was poured over the separation gel and a spacer (10-15 wells) was located at the top. Finally the gel cassette was left overnight at 4°C for advanced polymerisation.

*Table 8: Ingredients of the separation gel at different acrylamide concentrations.*

| | 6% | 8% | 10% | 12% |
|---|---|---|---|---|
| *Acrylamide/bisacrylamide (30:1)* | 4 | 5,3 | 6,7 | 8 |
| *Separation gel buffer* | 5 | 5 | 5 | 5 |
| *H2O* | 10,6 | 9,3 | 7,9 | 6,6 |
| *10%SDS* | 0,2 | 0,2 | 0,2 | 0,2 |
| *APS* | 0,2 | 0,2 | 0,2 | 0,2 |
| *TEMED* | 0,016 | 0,012 | 0,008 | 0,008 |
| *Total volume (ml)* | 20 ml | 20 ml | 20 ml | 20 ml |

*Table 9: Ingredients of the stacking gel for one gel cassette.*

| Volume µl | **4000** |
|---|---|
| *Acrylamide/bisacrylamide (30:1)* | 670 |
| *Stacking gel buffer* | 500 |
| *H2O* | 2700 |
| *10%SDS* | 40 |
| *APS* | 40 |
| *TEMED* | 4 |

#### 2.2.10.6 Western blotting of proteins

After the SDS PAGE, proteins were transferred on to a membrane (PVDF or Nitrocellulose). Because of the higher absorbance capacity, the nitrocellulose membrane was combined with a wet blot system.

The SDS gel and the nitrocellulose membranes were activated with $H_2O$ and equilibrated with 1x transfer buffer for 5 min. Two sheets of Whatman 3mm filter paper were cut and soaked in the transfer buffer as well. The gel was placed in between the filter paper and the membrane by avoiding any air bubbles. The membrane is covered with the second filter paper and this sandwich model is located in a wet blot cassette. The wet blot tank was filled with 1x transfer buffer and the cassette was placed in the tank. The transfer was carried out at 350 mA at 4°C for 75 min.

#### 2.2.10.7 Immunodetection of blots

Following the protein transfer onto membranes, these were washed for 10 min with TBS-$T_{20}$ on a horizontal shaker. The blots were stained with ponceu S (Sigma) for visualizing the protein bands and documented with an imager system (Intas).

Next, the blots were washed 3x 10 min with TBS-$T_{20}$ on a horizontal shaker. The unspecific binding sites on the membrane were saturated by TBS-$T_{20}$ (Blocking buffer) supplemented with 4 % BCA for 1 hr. Then the membranes were incubated overnight with appropriate dilutions of the primary antibody (see antibody list, Table 3) in blocking buffer at 4°C. After the antibody incubation, unbound antibody was removed by washing 3x 10 min with TBS-$T_{20}$ on a horizontal shaker. Then the membranes were incubated for 1 hr with appropriate dilutions of HRP linked secondary antibody (see antibody list, Table 4) at room temperature. Later, the membrane was washed again 3x 10 min with TBS-$T_{20}$ on a horizontal shaker. At the end of this step, the membranes were ready for incubation with ECL (Amersham) solution for 1 min. Finally, the membranes were wrapped into a foil of the autoradiography cassette and exposed to a x-ray film. The film was developed by an automatic photo developer (Agfa, Curix 60). After the development, the film was screened and digitalized with a scanner (Canon 5000F). The digitalized membranes were evaluated by a special software (AlphaEase FC) and the densitometric data were analyzed by a statistical analysis program (Graphpad prism).

### 2.2.11 RNA-DNA analytical methods

#### 2.2.11.1 Total RNA extraction from mammary glands and cell lines

The water used in the following protocols was pretreated with dimethyl pyrocarbonate (DEPC) which chemically inactivates contaminating RNases. DEPC was added to 0.1% and dissolved by vigorous shaking. The treated water was left overnight before being autoclaved.

#### 2.2.11.2 RNA extraction with "Trizol"

The method was used for large scale preparations of RNA from mammary glands of mice and cell lines. Before the Trizol treatment, the preparation of cells and mammary glands show differences. Cells were cultured in 6 well plates and incubated in various medium conditions. Therefore, prior to Trizol treatment the medium was discarded and cells were washed with ice cold PBS. Then, PBS was discarded and 1000 µl Trizol reagent was placed into each well scraped and then replaced into 2 ml eppendorf tubes.

The excised mammary glands were stored at -80 °C. Prior to the Trizol treatment, the whole gland was crashed in liquid nitrogen with a pestle and mortar. 50-100 mg pulverized tissue samples were placed into 2ml eppendorf tubes and homogenized in 1000µl Trizol reagent.

Samples were incubated for 5 minutes at room temperature to complete solubilisation. 200 µl of chloroform per 1000 µl Trizol was added, shaken vigorously for 15 seconds and incubated for 2-3 minutes at room temperature. Samples were centrifuged at 13000 rpm for 15 minutes at 4°C. The aqueous phase was transferred to a clean tube and RNA was precipitated by adding 500 µl isopropanol per 1000µl Trizol used and pelleted by centrifugation for 30 minutes at 8000 rpm at 4°C. The RNA pellet was washed once with DEPC treated 70% ethanol and air dried. The RNA was resuspended in 50 µl DEPC water.

#### 2.2.11.3 Determination of total RNA quality and quantity

RNA quality and quantity has a crucial role in gene expression analyses. Apart from traditional methods (spectrophotometry and gel electrophoresis), RNA quantification was performed with a bio-analyzer (Agilent 2100) using a microfabricated chip (Agilent RNA 6000 Nano). The system was able to report not only the amount of RNA but also the 260/280 nm ratio and the RNA integrity number (RIN).

RIN is a scale (from 1 to 10) to measure degradation and quality of RNA. Higher RIN value represents a better quality of RNA. Less than 9 RIN scaled samples were discarded from the experiments or were prepared again. Measurements were performed according to the manufacturer's (Agilent) recommendations.

### 2.2.11.4 Dnase treatment

Prior to cDNA synthesis, all crude RNA samples were routinely treated with DNase 1 (Turbo DNase) to remove possible genomic DNA contamination. This step not only eliminates remaining genomic DNA but also enhances PCR quality. DNase treatments were performed according to the manufacturer´s (Ambion) recommendations.

### 2.2.11.5 cDNA synthesis

Complementary DNA (cDNA) is synthesized from a mature mRNA template in a reaction catalyzed by reverse transcriptase. RT PCR is very useful to determine the gene expression in specific tissues or at different developmental stages. 1000 ng of total RNA was mixed with 0,5 µl of oligo $(dT)_{18}$ (10 pmol/µl) in a total volume of 12 µl to avoid possible secondary structure of the RNA, which might interfere with the synthesis. The mixture was heated to 70 °C for 10 min, and quickly chilled on ice. After a brief centrifugation the following reagents were added to the mixture:

5 µl 5 x buffer (Promega),

5 µl dNTPs 10µM (Biomol)

0,6 µl Rnasin (Promega)

1,4 µl DNAse, RNase free dd $H_2O$ (Biomol)

The content of the tube was gently mixed and incubated at 37 °C for 5 min. Then 1µl of reverse transcriptase enzyme (Promega MMLV) was added and further incubated at 37 °C for 1 hr for the first strand cDNA synthesis. Then, the reverse trancription was inactivated by heating 70°C for 15 min. Before the PCR run the volume is adjusted with DNAse, RNase free dd $H_2O$ to 200µl

### 2.2.11.6 Polymerase chain reaction (PCR)

The Polymerase Chain Reaction is a powerful technique that is widely used for amplification of specific DNA sequences in vitro by using appropriate synthetic oligonucleotides (primers). The general principle of PCR starts from a pair of oligonucleotide primers that are designed so that a forward primer directs synthesis of DNA towards a reverse primer, and vice versa. During the PCR, Taq polymerase which is purified from bacterial thermos aquaticus and is a heat stable enzyme, catalyses the synthesis of a new DNA strand that is complementary to a template DNA from the 5´ to 3´ direction by a primer extension reaction, resulting in the production of the DNA region flanked by the two primers. It allows the rapid and unlimited amplification of specific nucleic acid sequences that may be present at trace amount concentrations in complex mixtures.

### 2.2.11.7 PCR for genotyping mice

The following protocol was performed for the determination of the human ET-1 transgene from mouse tail biopsies (see isolation of genomic DNA from tissue) (Table 10).

*Table 10: Ingredients of the PCR mastermix and PCR running program # dd$H_2O$= DNAse, RNase free dd $H_2O$, ## Template= Genomic DNA*

**Master mix**

| Ingredients | Conc. | Amounts (µl) |
|---|---|---|
| 10x PCR Buffer | - | 5 µl |
| MgCl2 | 25 mM | 4 µl |
| dNTPs | 10µM | 1 µl |
| Forward Primer | 0,2 µM | 2 µl |
| Reverse Primer | 0,2 µM | 2 µl |
| Taq Polimerase | | 0.3 µl |
| dd$H_2O^{\#}$ | - | 31,2 µl |
| Template$^{\#\#}$ | 20 ng | 5 |
| Volume | - | 50 |

**Thermal profile**

| Steps | Temp. (°C) | Time (sec.) | Return | Cycle |
|---|---|---|---|---|
| 1 | 94 | pause | | |
| 2 | 94 | 6 | | |
| 3 | 60 | 30 | | |
| 4 | 72 | 2 | | |
| 5 | 94 | 30 | 3 | 9 |
| 6 | 60 | 30 | | |
| 7 | 72 | 600 | | |
| 8 | 4 | pause | | |

The reactions were carried out in a PCR thermocycler (Biometra) by employing a Taq polymerase kit (Invitek). PCR products (~500 kb) were separated by agarose gel electrophoresis and monitored with an UV gel imager system (Intas).

### 2.2.11.8 PCR for sample control and optimisation

Each PCR reaction has different primer annealing and melting temperatures and primer specificity characteristics. The concentration of the various components and the annealing temperature can be altered to improve the overall yield, fidelity and specificity of the reaction. In general, the above conditions were based on a $MgCl_2$ concentration of 1.5 µM and a dNTP concentration of 250 µM, when necessary these conditions were varied to optimise the amplification. The annealing temperature was optimised for each primer pair using a temperature gradient (see Table 2).

The PCR conditions are dependent on the nature of the template DNA and the primers involved. In general, the oligonucleotide primers were designed with similar melting temperatures. This PCR application represents also a last check point for cDNA quality prior to real-time PCR (Table 11). PCR products were separated by agarose gel electrophoresis and monitored with a UV gel imager system (Intas)

*Table 11: Ingredients of the PCR mastermix and PCR running program.*Variable annealing temperatures see (Table1) **Extension temperature designed from the recommendations of the manufacturers of Taq polymerase (Eppendorf)*

**Master mix**

| Ingredients | Conc. | Amounts (µl) |
|---|---|---|
| 10x PCR Buffer | - | 2,5 µl |
| MgCl2 | 50 mM | 0,5 µl |
| dNTPs | 10µM | 1 µl |
| Forward Primer | 0,2 µM | 0,5 µl |
| Taq Polimerase | 0,2 µM | 0,5 µl |
| dd $H_2O$ | | 15 µl |
| Template | 5 ng /µl | 5 µl |
| Volume | - | 25 µl |

**Thermal profile**

| Steps | Temp. (°C) | Time (sec.) | Return | Cycle |
|---|---|---|---|---|
| 1 | 94 | 180 | | |
| 2 | 94 | 15 | | |
| 3 | 58-62 * | 30 | | |
| 4 | 68 ** | 30 | 2 | 39 |
| 5 | 72 | 300 | | |
| 6 | 4 | pause | | |

### 2.2.11.9 Real-time PCR

Real time PCR or quantitative PCR is a variation of the standard PCR technique used to amplify and simultaneously quantify a targeted DNA or messenger RNA (mRNA) molecule. It enables both detection and quantification (as absolute number of copies or relative amount when normalized to DNA input or additional normalizing genes) of one or more specific sequences in a DNA sample. The syber green binds to all double-stranded DNA during PCR, causing a fluorescence signal. An increase in the DNA product during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified.

Amounts of RNA or DNA are then determined by comparing the results to a standard curve produced by real-time PCR of serial dilutions of a known amount of RNA or DNA.

The technology is employed to provide quantitative measurements of gene transcription which monitorizes the changes on the expression of the particular gene between the ET-1 transgenic mice and wild type mice or the response of cell cultures to the administration of different biochemical agents.

*Table 12: Ingredients of the PCR mastermix and PCR running program *Variable annealing temperatures see Table 2.*

**Master mix**

| Ingredients | Conc. | Amounts (µl) |
|---|---|---|
| 2x sybergreen master mix | - | 12,5 µl |
| Forward Primer | 0,2 µM | 0,5 µl |
| Taq Polimerase | 0,2 µM | 0,5 µl |
| dd $H_2O$ | | 6,5 µl |
| Template | 5 ng /µl | 5 µl |
| Volume | - | 25 µl |

**Thermal profile**

| Steps | Temp. (°C) | Time (sec.) | Return | Cycle |
|---|---|---|---|---|
| 1 | 95 | 600 | | |
| 2 | 94 | 15 | | |
| 3 | 58-62 * | 30 | | |
| 4 | 72 | 30 | 2 | 39 |
| 5 | 72 | 300 | | |
| 6 | 4 | pause | | |

Primer efficiency, standard curve and expression analyses was performed with the software Relative Expression Software Tool (REST 2008) which is using Pair Wise Fixed Reallocation Randomisation Test for statistical analyses (Pfaffl, et al., 2002).

#### 2.2.11.10 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for the separation of DNA fragments or PCR products. TAE buffer is used as electrophoresis buffer and a 1% agarose gel is prepared with the same buffer. In order to dissolve the agarose, the buffer was boiled in a microwave oven for 1-2 min. Then the agarose gel was cooled down (about 60°C) on the bench for 5 min. The DNA is visualised in the gel by addition of ethidium bromide (EtBr). Hereby 0.8% EtBr dye is added to the agarose and swirl to mix. The liquid agarose is slowly poured into the gel cassette and the combs were inserted. Finally the gel is cooled down for 30 min.

Before loading, samples were mixed with loading buffer. The samples were loaded in to the wells of the gel and electrophoresis was carried out at a steady voltage (110 V) for 20 min. Finally, the gel was documented with an UV gel imager system (Intas).

# 3 RESULTS

## 3.1 Basic characterization of ET-1 transgenic mice

### 3.1.1 Identification of the ET-1 transgene

To verify the genetic status of the animals, the presence of the transgene was assessed by PCR genotyping of tail biopsies. Figure 17 displays the analysis of the PCR products using transgene specific primers from the various study groups.

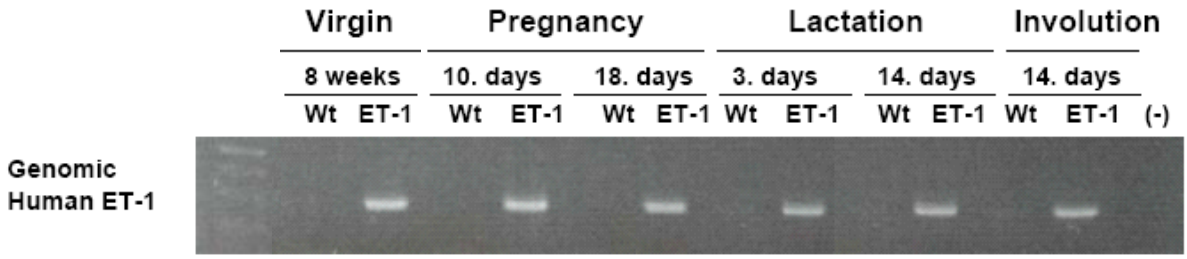


*Figure 17: Identification of the human ET-1 transgene in mice from different experimental groups*.

ET-1 transgenic mice were successfully identified. The presence of the transgene was proven by employing appropriate primers specific for the human ET-1. In figure 17 the samples were arranged into 4 main study groups according to different stages of the mammary gland development (virgin, pregnancy, lactation and involution).

### 3.1.2 Expression pattern of the transgene during mammary gland development

Gene expression of ET-1 in the mammary glands of the 4 study groups mentioned above was assessed by RT-PCR using primers designed to either specifically amplify the human or both the human and mouse transcript. Therefore, total RNA was obtained from homogenized whole mammary glands using liquid nitrogen and cDNA was synthesized. Samples in which the reverse transcriptase was omitted served as negative controls. cDNA obtained from the human mammary gland cell line MCF 7 served as a positive control for the human transcript (Figure 18).

As shown in figure 18, the human ET-1 transgene was only detected in mammary glands of pregnant and lactating animals (Figure 18A), demonstrating that expression of the transgene was restricted to these physiological stages.

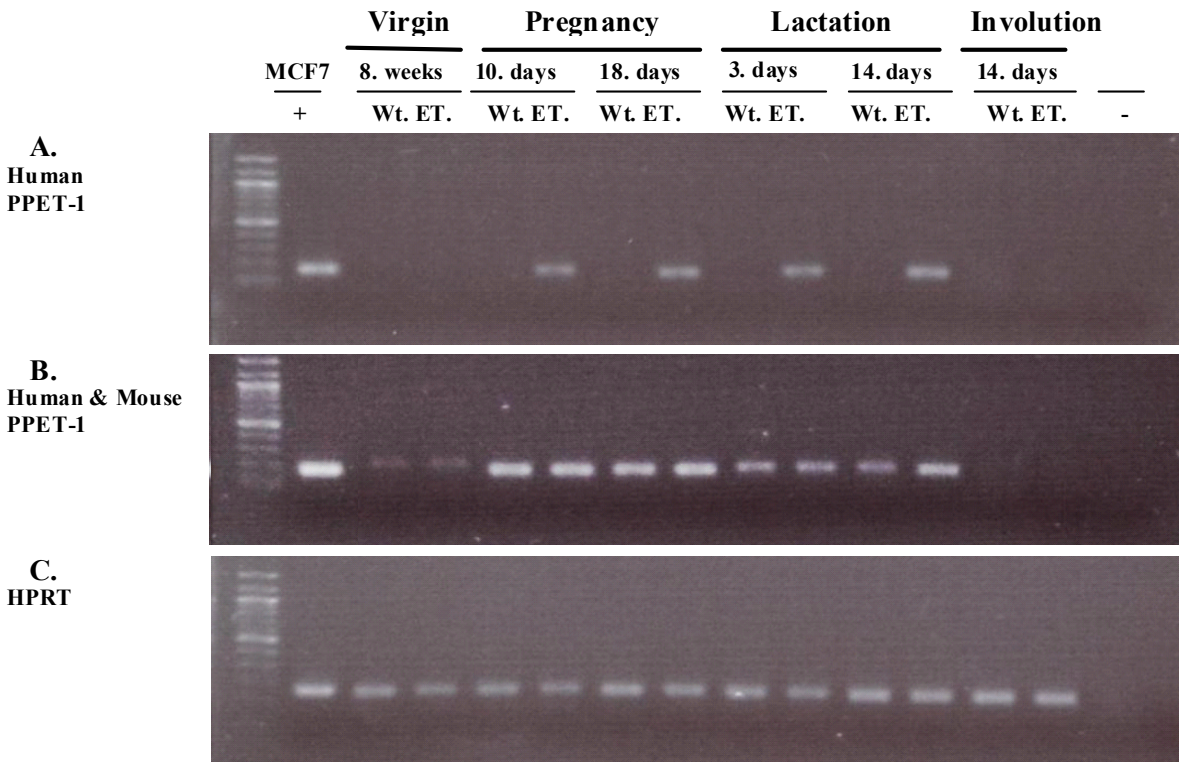


*Figure 18: ET-1 expression analysis in the various physiological stages of mammary gland development. (A) A specific primer set was used to demonstrate human ET-1 transgene expression (B) human and mouse ET-1 gene expression, (C). HPRT gene expression serving as a loading control.*

In contrast, using primers recognizing both the transgenic (human) and the endogenous (mouse) ET-1, a signal was detected in 8 weeks old virgin mice, (Figure 18 b), suggesting that the expression pattern of the endogenous ET-1 gene was different from that of the transgene

Since human ET-1 was only detected in mammary glands from pregnant and lactating animals, the expression level of total ET-1 in mammary glands of those groups of animals was assessed by real-time PCR. As shown in Figure 18b, total endothelin expression was increased to about six fold in transgenic mice compared to wild type (Wt) mice during pregnancy as well as lactation, demonstrating significantly higher expression levels in these physiological stages (Figure 19). Therefore, this animal model is suitable for the analysis of the role of an increased ET-1 expression with the mammary gland at these stages.

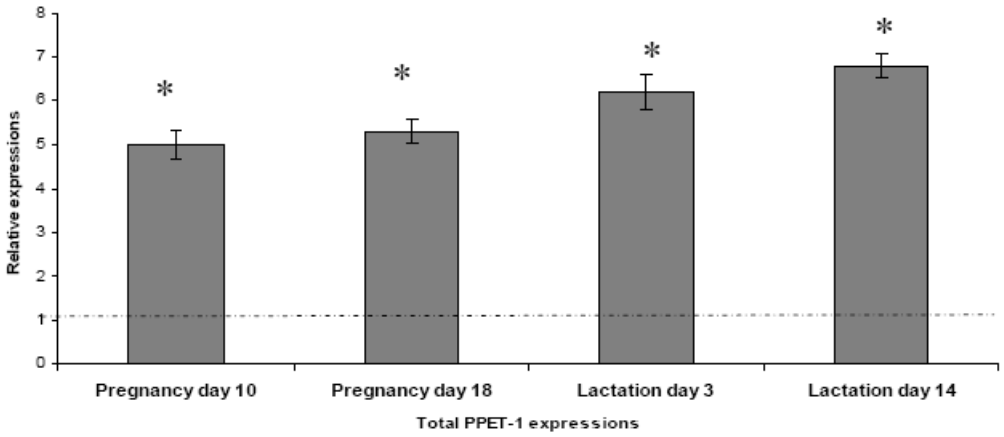


*Figure 19: Relative total endothelin expression levels in transgenic animals. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). * P<0,05 n=4 females for each group*.

### 3.1.3 Expression of endothelin receptors

ET-1 acts through its two G-protein coupled receptors, the ETAR and the ETBR. To determine whether overexpression of ET-1 in transgenic animals was accompanied by changes in the expression levels of these two receptors, expression of both receptors in the mouse mammary glands of day 3 lactating wild type and transgenic ET-1 mice was determined at the mRNA and the protein level by real-time PCR and western blotting, respectively.

Based on the histological differences of the transgenic group compared to wild type mice at lactation day 3, in Figure 20 the relative mRNA expression for the endothelin receptors were analyzed. The mRNA expression of the ETAR and ETBR in ET-1 transgenic mice did not differ from wild type animals. Additionally, this result was confirmed at the protein level for the two receptors (Figure 21).

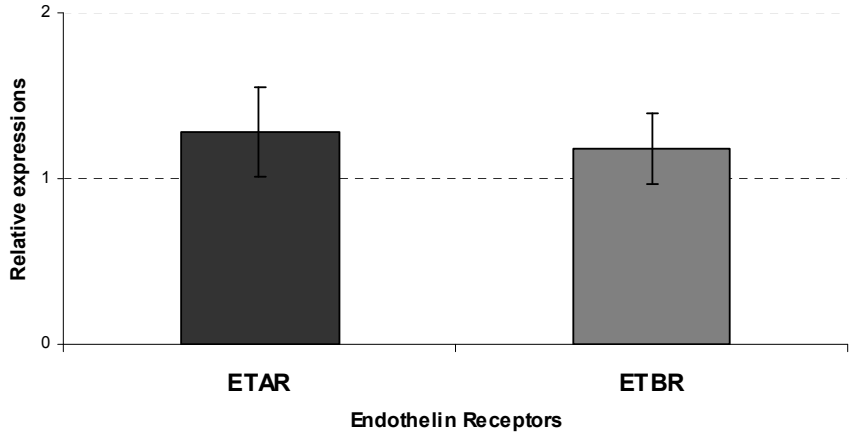


Figure 20: Analysis of the relative mRNA expression levels of the two ET-1 receptors, ETAR and ETBR, in mammary glands at lactation day 3 employing real time PCR. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). n=4 females for each group no significant difference was observed.

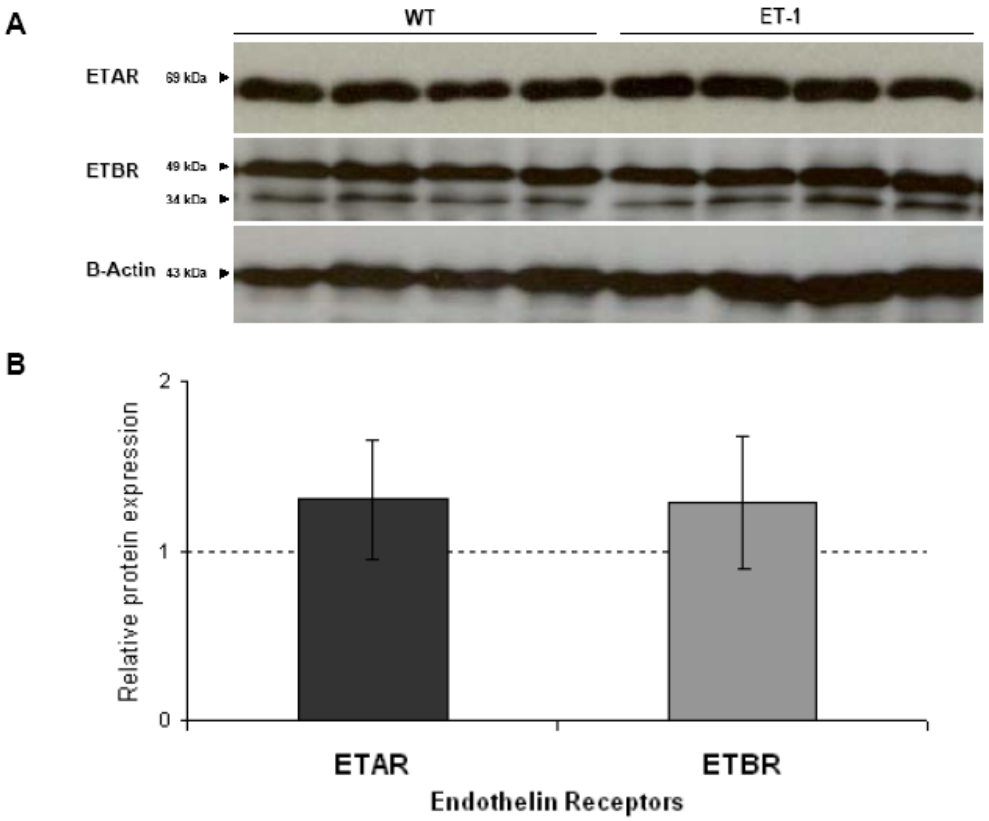


*Figure 21: A. Western blot analysis of the two ET-1 receptors ETAR (69kDa) and ETBR (49/34kDa) in mammary glands of wild type and transgenic mice. Beta-actin (43kDa) served as loading control. B. Densitometric analysis of endothelin receptors at lactation day 3, densities of the receptors were normalized with Beta-actin density n=4 females for each group.*

## 3.2 Impaired lactational competence in ET-1 transgenic females

When homozygotic ET-1 transgenic females gave birth, it was noted that some of the newborns died very soon after birth. Macroscopically, a lack of milk in the stomach was found in several pubs, indicating a lactational deficit.

To validate these findings, mortality and growth rates of the offspring was monitored and the numbers of newborns, successfully raised mice and animals reaching adulthood was recorded (Table 13). Additionally, the weight gain of newborns over the lactational period was monitored (Figure 22A).

*Table 13: Neonatal survival: Dead born wild type: 0.2 % ET-1: 5.6 %; Neonatal death wild type: 0.4%, ET-1: 5.4 %; Survival at the end of lactation wild type: 99.4%, ET-1:89 %. (Total number of animal wild type=894, ET-1=663) Fisher exact test has been performed for the statistical analysis*

| | **WILD TYPE** *(%)* | **ET-1** *(%)* | **P** |
|---|---|---|---|
| Dead born *(day 1)* | 2 *(0,2)* | 37 *(5,6)* | P<0,0001 |
| Neonatal death *(days 1-20)* | 4 *(0,4)* | 36 *(5,4)* | P<0,0001 |
| Survival at the end of lactation *(day 20)* | 888 *(99,4)* | 590 *(89)* | |
| Total | 894 *(100)* | 663 *(100)* | |

When assessing the weight gain of the offspring, it was noted that pups suckled by transgenic females gained significantly less weight than those suckled by wild type females. Whereas the initial body weight at birth did not differ between the two genetic groups, pups suckled by transgenic mothers displayed a significantly lower weight gain during the first 11 days. After that, the weight gain slope of transgenic animals became comparable to wild type females. However, when the mice reached adulthood, differences in body weight had disappeared (Figure 22 B).

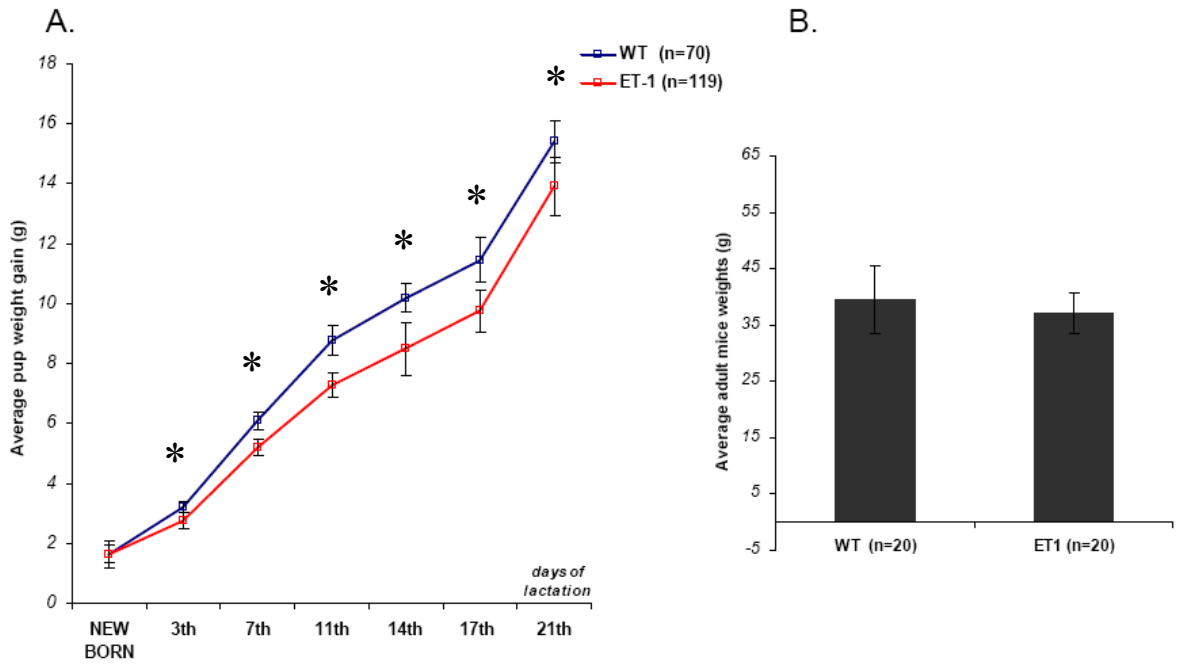


*Figure 22: A) Measurement of the average weight gain of newborn mice derived from wild type and transgenic females through the entire lactational period.*P<0.05 (Student´s T-test). B) Weight of adult wild type and transgenic mice; no statistical difference between the groups.*

## 3.3 Histopathology

As increased mortality and reduced weight among pups suckled by transgenic mothers point to impaired lactational competence of transgenic females, a morphological analysis of the mammary glands was performed in these animals. For histopathological analysis, whole mount preparations and HE stained tissue sections of mammary glands from wild type and transgenic females were compared. The specimens were obtained from 10$^{th}$ and 18$^{th}$ day pregnant females in order to characterize ductal developmental and ductal differentiation, respectively. Samples from 3$^{rd}$ and 14$^{th}$ day lactating animals were collected to analyze the structure of lactating alveoli.

### 3.3.1 Pregnancy day 10

ET-1 transgenic mammary glands were characterized by extensive ductal branching and formation of alveoli in the mammary fat pad as analysed by whole mount staining (Figure 23 c-d) and this is comparable to wild type glands (Figure 23 a-b). In HE stained samples the alveolar structure and distribution, as well as the amount of surrounding fat did not differ in ET-1 transgenic whole mounts compared to wild type as shown in figure 24.

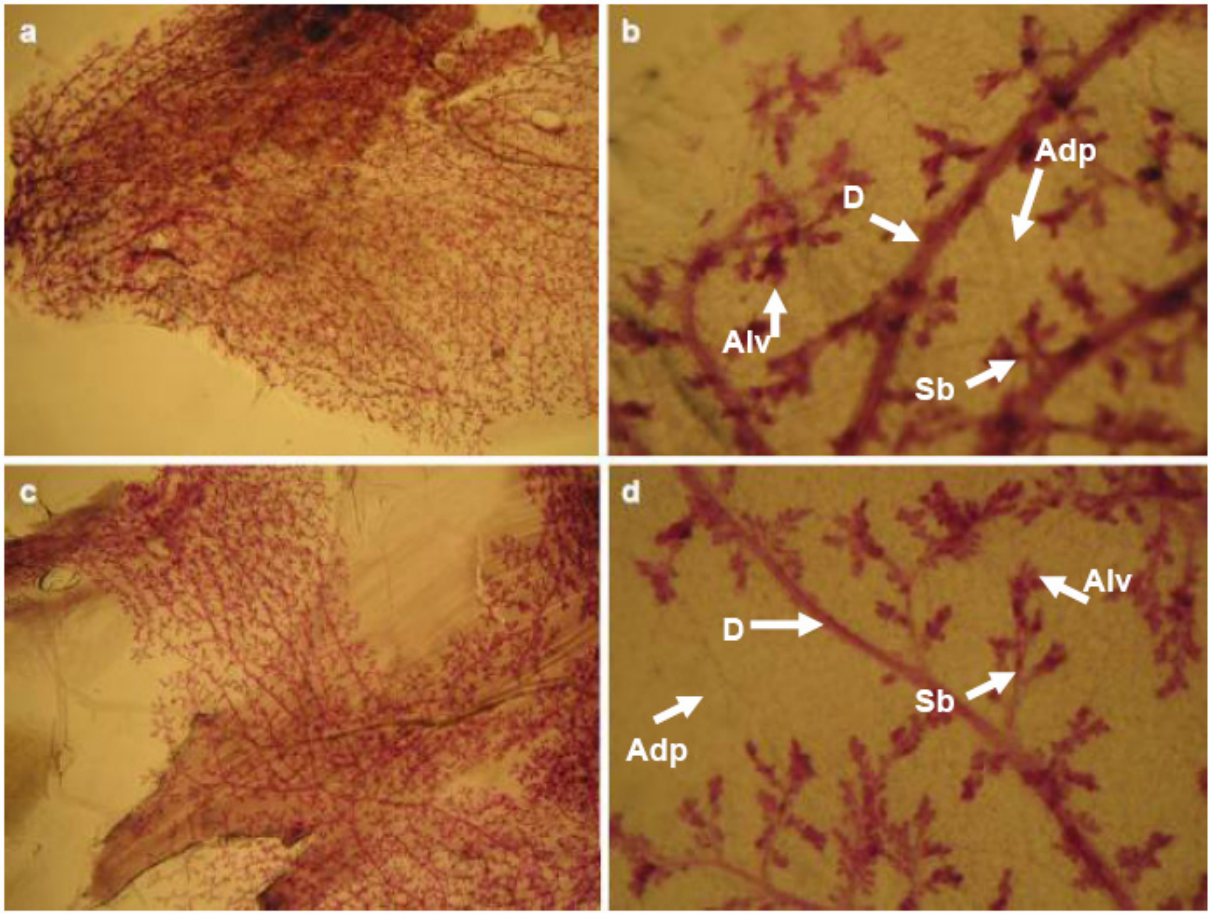


*Figure 23: Whole mount analysis of mammary gland structures of wild type and ET-1 transgenic mice at pregnancy day 10: Growing ductus (D) through the fat pad (Adp). Wild type: a; ET-1: c (x 11 magnifications), wild type: b; ET-1: d (x 63 magnifications). Ductal growth involves lengthening and branching of the epithelium (Sb). In parallel, alveolar lobules are beginning to develop (Alv) at pregnancy day 10.*

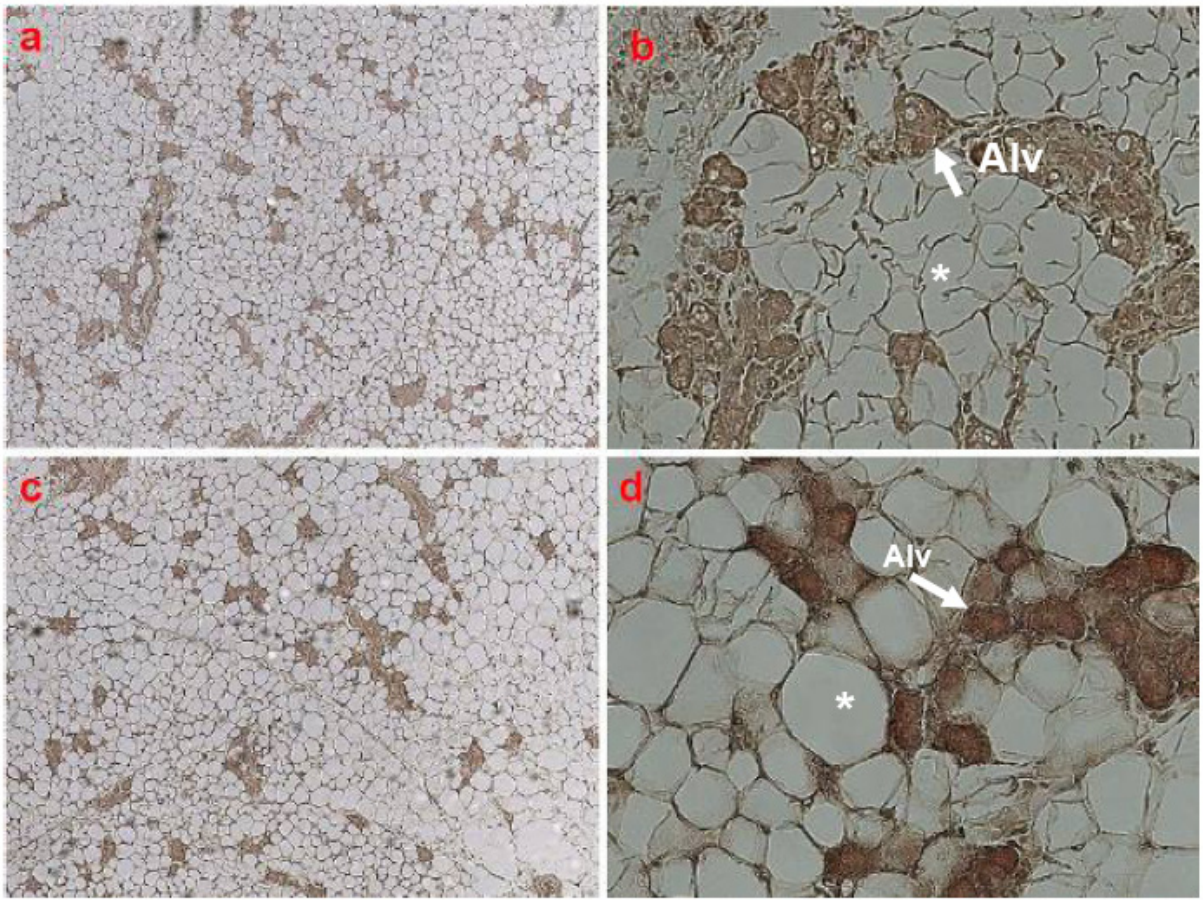


*Figure 24: Histological features of the mammary gland at pregnancy day 10 in wild type and ET-1 transgenic mice. All sections have been stained with haematoxylin and eosin (HE). Alveolar lobules (Alv) are beginning to develop into the fat pad. Adipocytes (*) are prominent in the mammary gland. Wild type: a; ET-1: c (x 50 magnifications), wild type: b; ET-1: d (x 200 magnifications).*

### 3.3.2 Pregnancy day 18

As pregnancy proceeds, substantial proliferation of the alveolar buds with the formation of lobuloalveolar units occurs that is accompanied by a progressive loss of fat cells within the mammary stroma. As shown in figure 25, both wild type and transgenic animals exhibited vastly expanded mammary epithelium within the stroma.

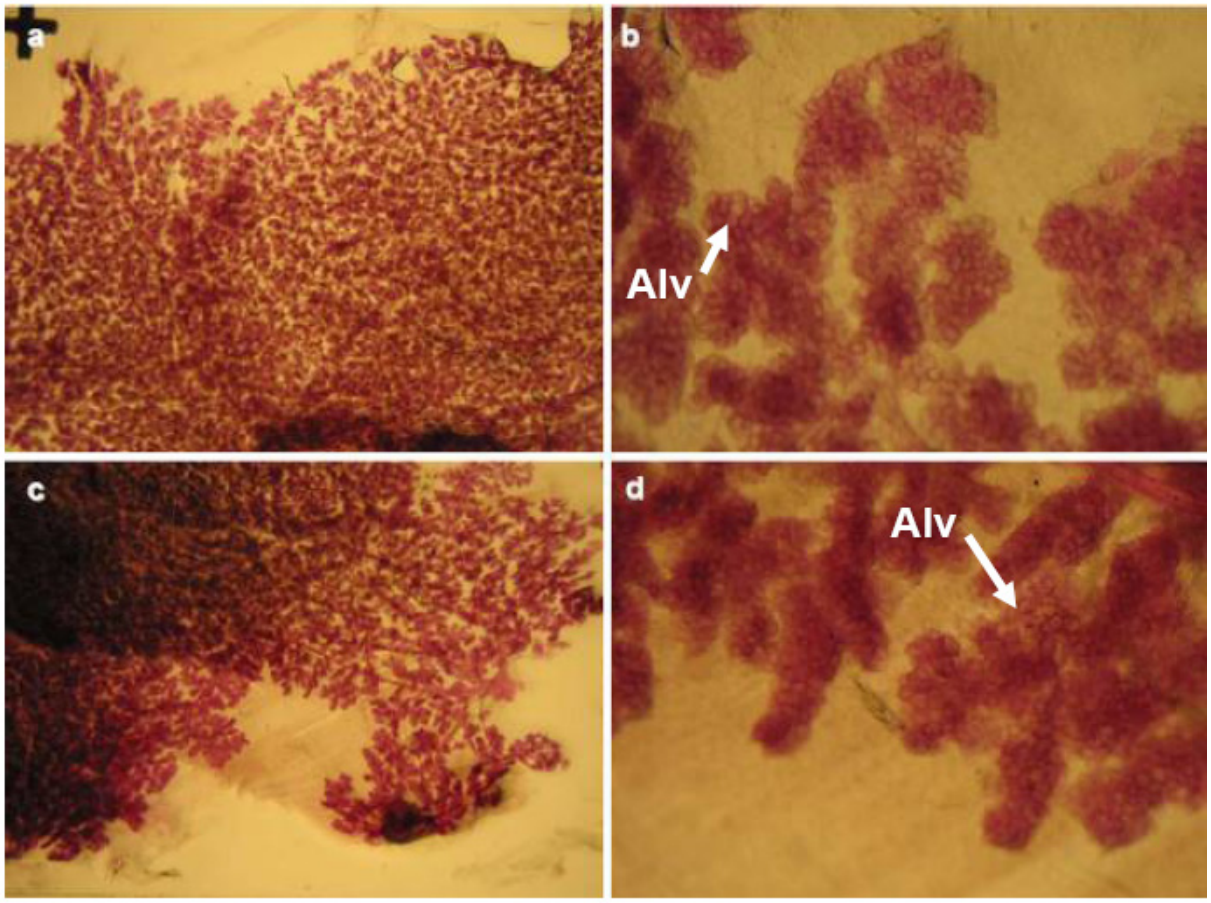


*Figure 25: Whole mount analysis of mammary gland structures of wild type and ET-1 transgenic mice at pregnancy day 18: wild type: a; ET-1: c (x 11 magnifications), wild type: b; ET-1: d (x 63 magnifications). Ductal outgrowth reaches the tissue border of the fat pad and arrows indicate the alveoli (Alv) that have filled the majority of the fat pad. Grape shaped alveolar structures characterise the functional differentiation at pregnancy day 18.*

When assessed by HE staining, cytoplasmic lipid droplets (CLD) are characteristic features of the late phase of pregnancy (Anderson, et al., 2007). Such CLDs indicating secretory differentiation were found both in wild type and ET-1 transgenic mammary glands at the late stage of pregnancy. Distribution of the epithelial cells within the fat pad and density of the epithelial cells did not differ significantly between both groups (Figure 26). Therefore, the results obtained so far demonstrate that ET-1 overexpression does not provoke any histological alterations of the mammary gland in pregnant animals, suggesting that differentiation of the mammary epithelium was not affected in transgenic animals at this developmental stage.

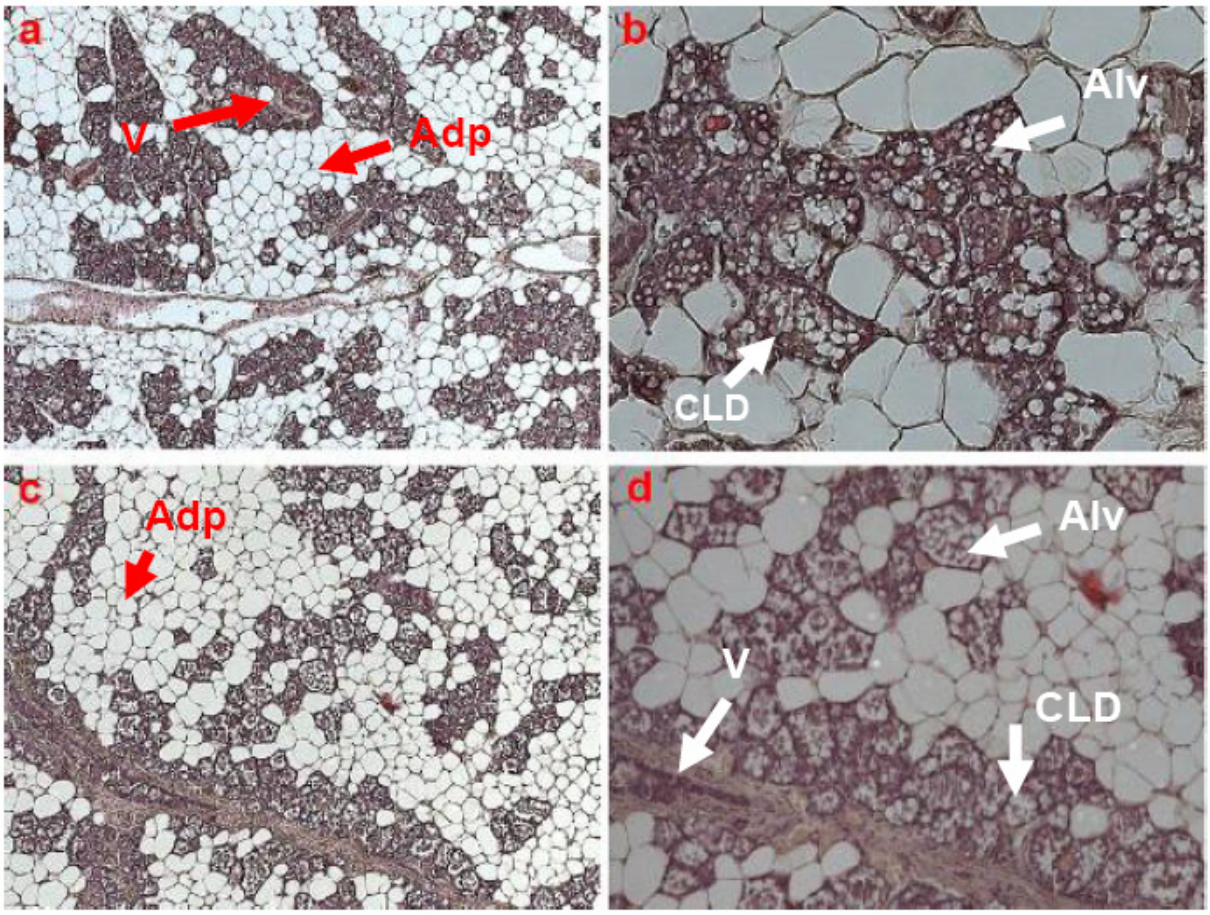


*Figure 26: Histological features of the mammary gland at the pregnancy day 18. All tissue sections were stained with HE. Wild type: a; ET-1: c (x 50 magnifications), wild type: b; ET-1: d (x 200 magnifications). As depicted here both animal groups demonstrated the same histological architecture. The alveoli have continued to develop and have filled the majority of the fat pad. There are still areas of fat within the gland that will not disappear until lactation. The epithelial cells have begun to produce milk proteins and lipid (Alv). The luminal spaces of the alveoli are filled by substance and cytoplasmic lipid droplets (CLD) and the individual alveoli are surrounded by blood vessels (V).*

### 3.3.3 Lactation day 3

Lactation is a period of continuous milk production. By the suckling stimuli stored milk is removed from the alveoli. Due to milk accumulation, the alveolar epithelium expands through the adipocyte and adipocytes lose their contents (Oakes, et al., 2006).

In wild type mammary glands the entire fat pad is filled with well expanded alveoli demonstrated by the dense staining patterns of the glands (Figure 27 a, b). In contrast, in the ET-1 transgenic females, areas with poor alveolar expansion were found where alveolar size and density of the mammary glands resembled a morphology seen in the late phase of pregnancy instead of lactation (Figure 27 c, d).

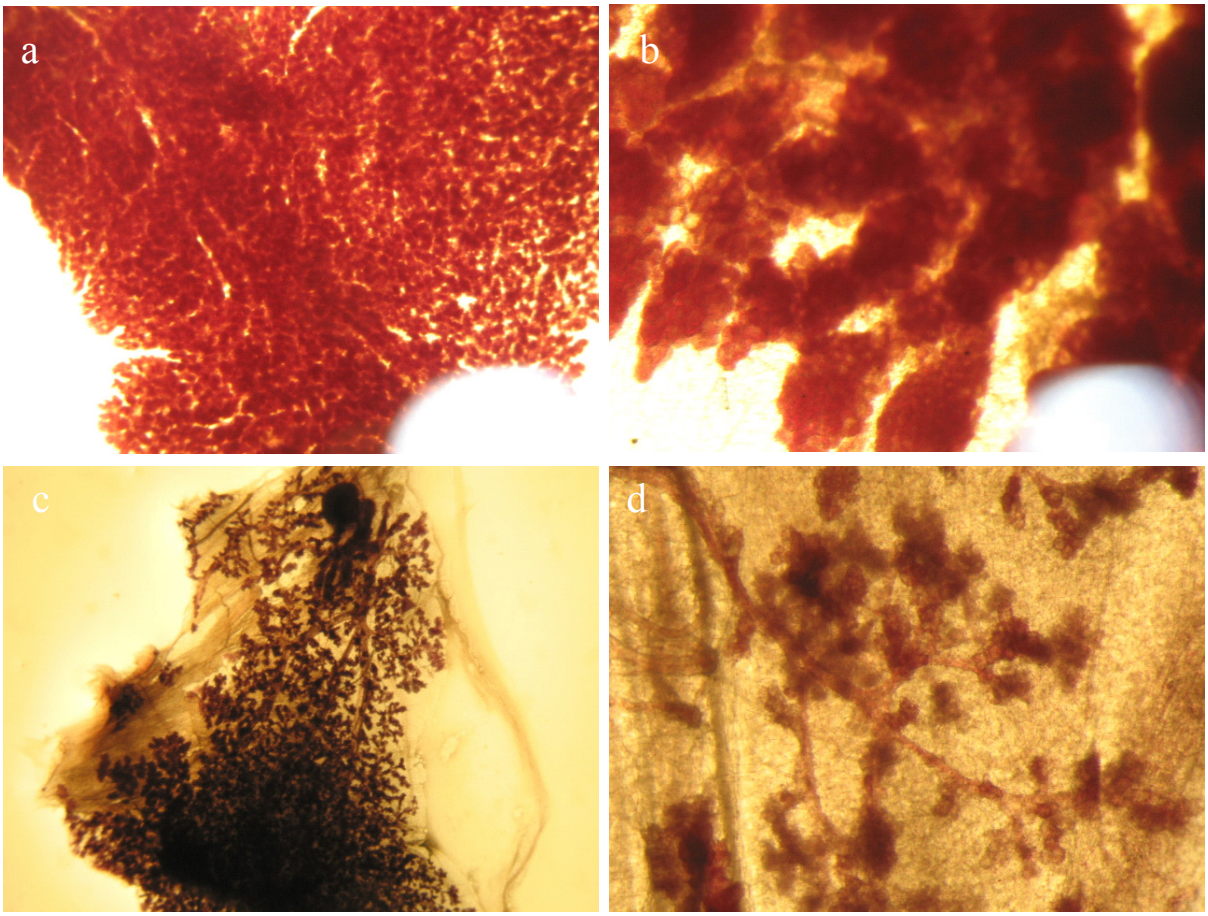


*Figure 27: Whole mount analysis of mammary gland structures of wild type and ET-1 transgenic mice at lactation day 3. Wild type: a; ET-1: c (x 11 magnifications), wild type: b; ET-1: d (x 63 magnifications). Wild type mice exhibited a well expanded alveolar architecture (a, b). In contrast, density of ducts, the extent of ductal branching and alveolar formation in ET-1mammary gland was reduced or regressed compared to wild type mammary glands (c, d).*

The histology of the mammary gland at this time point is generally characterised by the presence of fully expanded lumens surrounded by a densely stained layer of epithelial cells. In wild type mammary glands, the accumulation of milk fully expanded the alveoli and a relatively small volume of adipose tissue was present (Figure 28 a, b). In contrast, ET-1 transgenic glands displayed focal areas with collapsed alveoli and persistence of intracellular lipid

droplets. In addition, the amount of adipose tissue was increased in these animals. These alterations are defined as precocious involution (Figure 28 c, d).

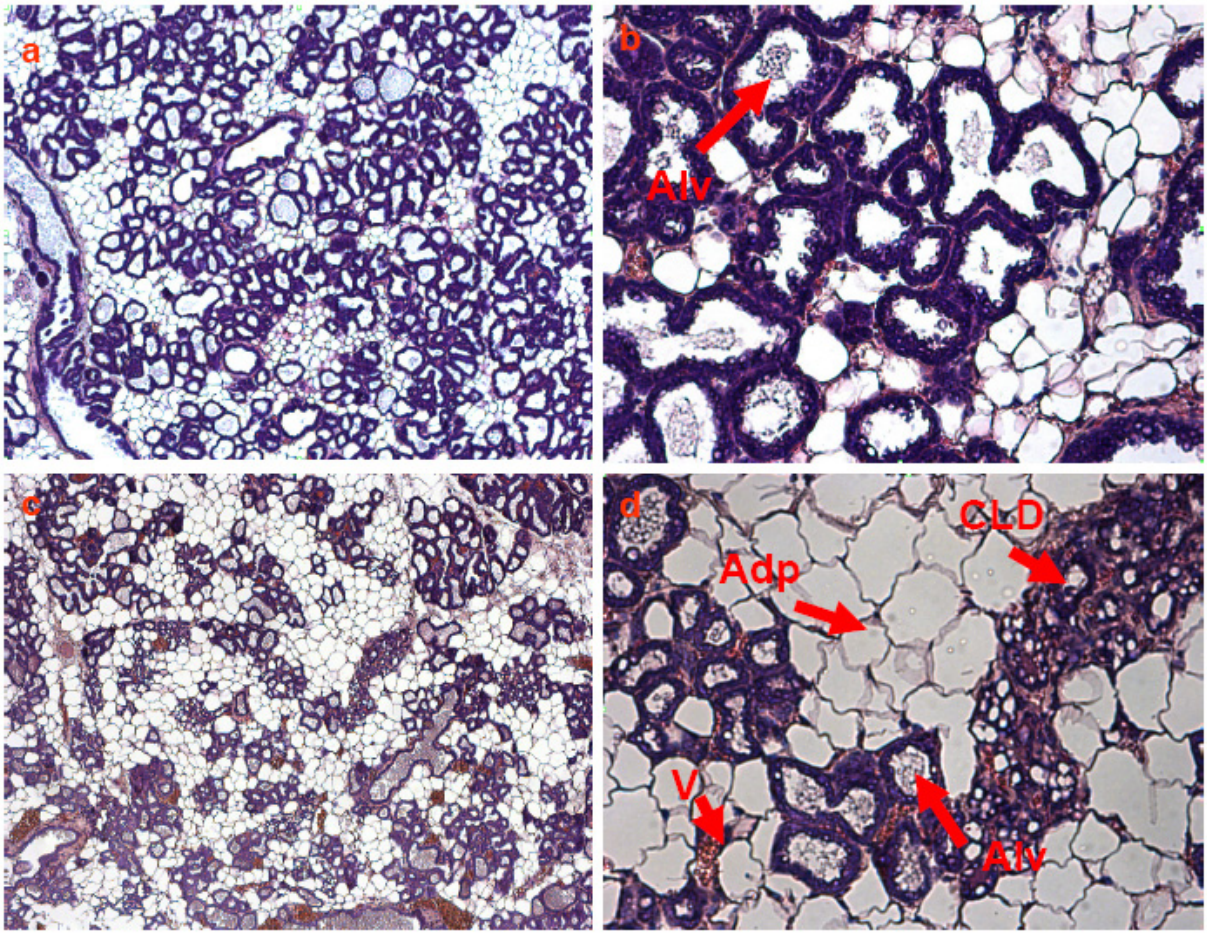


*Figure 28: Histological features of the mammary gland at the lactation day 3. All tissue sections stained with HE. Wild type: a; ET-1: c (x 50 magnifications), wild type: b; ET-1: d (x 200 magnifications). After parturition and with the onset of lactation the fat in the adipocytes is metabolized and the alveoli expand to fill the gland completely (a,b). Red blood cells indicating blood vessels (V) and a thin layer of connective tissues are visible between the alveoli. However, histological features of ET-1 transgenic glands (c, d) resemble late pregnancy with the presence of cytoplasmic lipid droplets (CLD) and poorly expanded alveoli reflecting a secretory defect.*

#### 3.3.3.1 Measurement of alveolar expansion

As outlined above, the histological analysis of the transgenic animals pointed to a reduction of alveolar size after parturition. To validate this finding on a quantitative level, alveolar diameters were measured using a Zeiss axiovison software. Hereby, 10 slides (5 slides for wild type and 5 slides for ET-1) have been recorded digitally. Four random pictures were taken from each slide and analysed by counting.

Considering the postpartum proliferation of the mammary glands, both groups exhibited an equal amount of alveoli. However, the size of the alveoli in the transgenic group (mean alveolar size: ~220 $\mu m^2$) was found to be relatively smaller than in the wild type group (mean alveolar size: ~400 $\mu m^2$, Figure 29A). In order to evaluate the distribution of alveoli in the

mammary glands of the two genetic groups, the mean dimension of one alveolus was determined first by doing the calculations based on the analysis of 6930 alveoli (3596 from wild type glands and 3334 from transgenic glands). The mean diameter is ~300 $\mu m^2$ which has been considered as reference for the following analysis of the two groups. 75% of the total number of alveoli are smaller than the average value of 300 $\mu m^2$ in ET-1 transgenic mice. However, in the wild type group, less than 50 % of the alveoli were smaller than the average value of the alveolar size (Figure 29B).

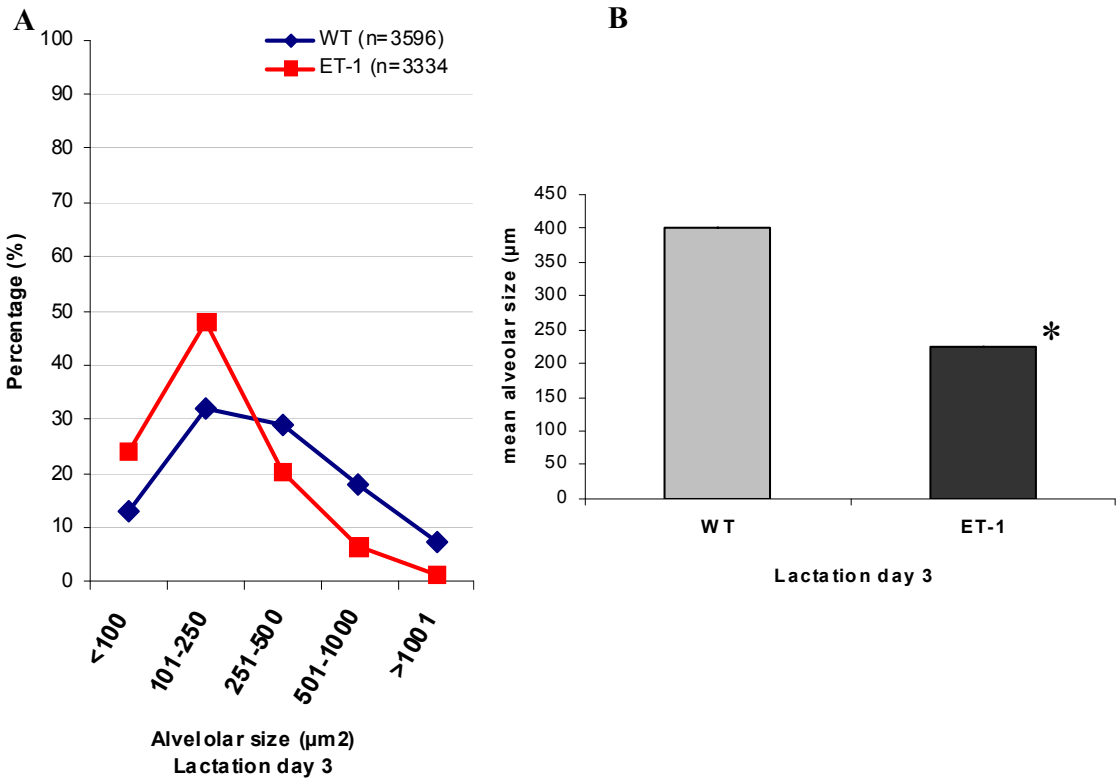


*Figure 29: With the start of lactation the fat in the adipocytes is metabolized and the alveoli expand to fill the gland. Expansion of the alveolar size reflects the secretory activity in the mammary glands A. Alveolar size and distribution of alveoli in the mammary glands according to their size in percentage, B. Average alveolar size wild type: 402µm², ET-1: 223 µm², (*): p< 0.05 Student's T-test).*

### 3.3.4 Lactation day 14

The ET-1 transgenic group exhibited a phenotype with a decreased weight gain of the pups during the first half of lactation. On the other hand, throughout the second half of lactation, suckling pups of the transgenic group exhibited a parallel and comparable weight gain slope compared to wild type animals. This finding suggests that the transgenic mammary glands were able to sustain the lactation and continue to nourish their youngs and suspend the precocious involution. Therefore lactation day 14 becomes important to analyse how transgenic animals compensate the secretory defect throughout lactation. At lactation day 14, the mammary glands reached the maximal secretory capacity reflecting the achievement of milk

production (Kozakai, et al., 2002). In figure 30, the mammary glands of wild type animals represented well extended alveoli and milk was visible in alveoli. However ET-1 transgenic mammary glands exhibited a loss of density of the alveoli.

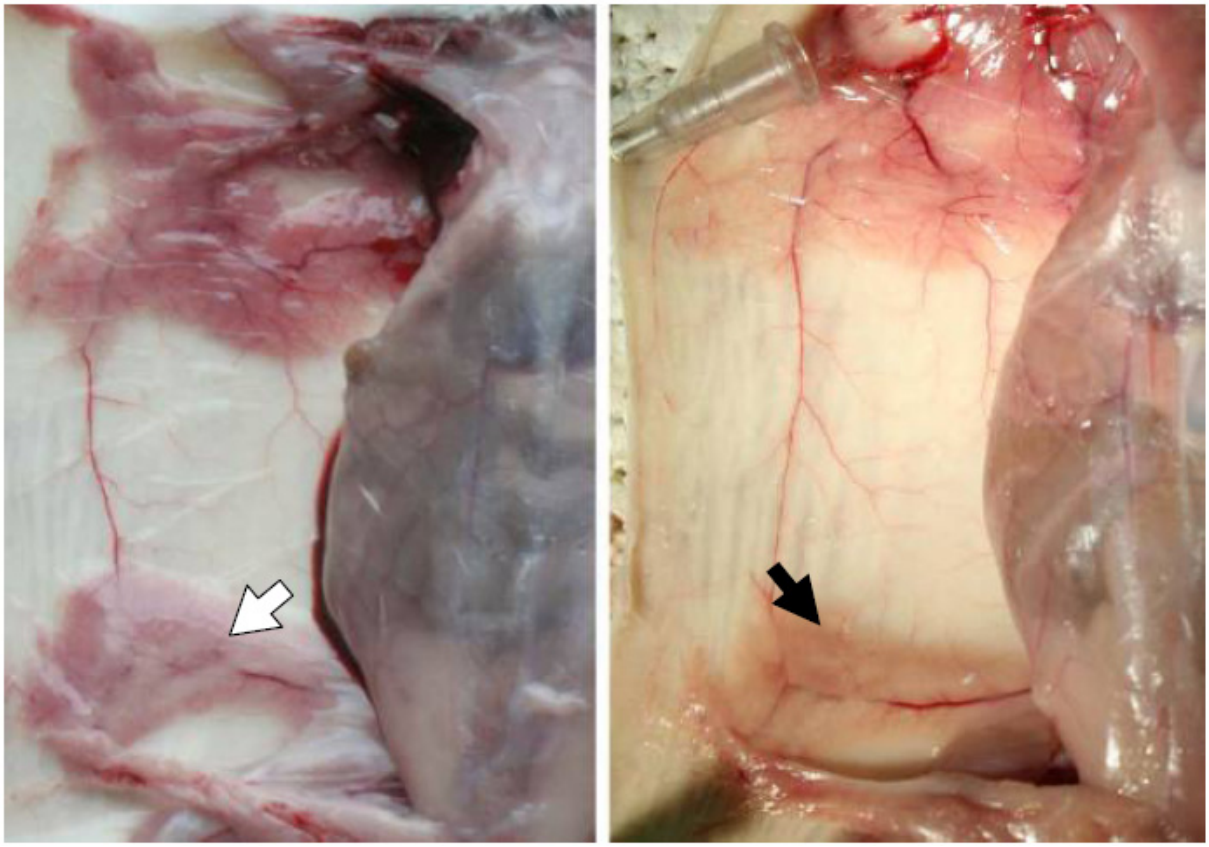

*Figure 30: Macroscopical appearance of the mammary glands at the lactation day 14. Left: wild type, normal lactating and well expanded mammary gland, the milk is visible (pointed with white arrow); right: ET-1 transgenic, macroscopically several irregularities such as shrinkage of the mass of the gland and lack of milk can be seen (pointed with black arrow).*

Hematoxylin/eosin staining revealed an increase in adipose tissue, in which collapsed secretory alveoli were found (Figure 31 c1,d1). These histological changes were reminiscent of the morphology seen during involution of the mammary gland (Richert, et al., 2000). In addition to this abnormality, some parts of the mammary gland displayed intense lobular proliferation with cells characterized by increased cytoplasmatic volume and enlarged nuclei (Figure 31 c-2 d-2). Although no definite signs of pathological dysplasia were detected, alveolar organization was lost in parts of these areas. Therefore, immunohistostaining of smooth muscle actin was employed in order to rule out an underlying malignant transformation.

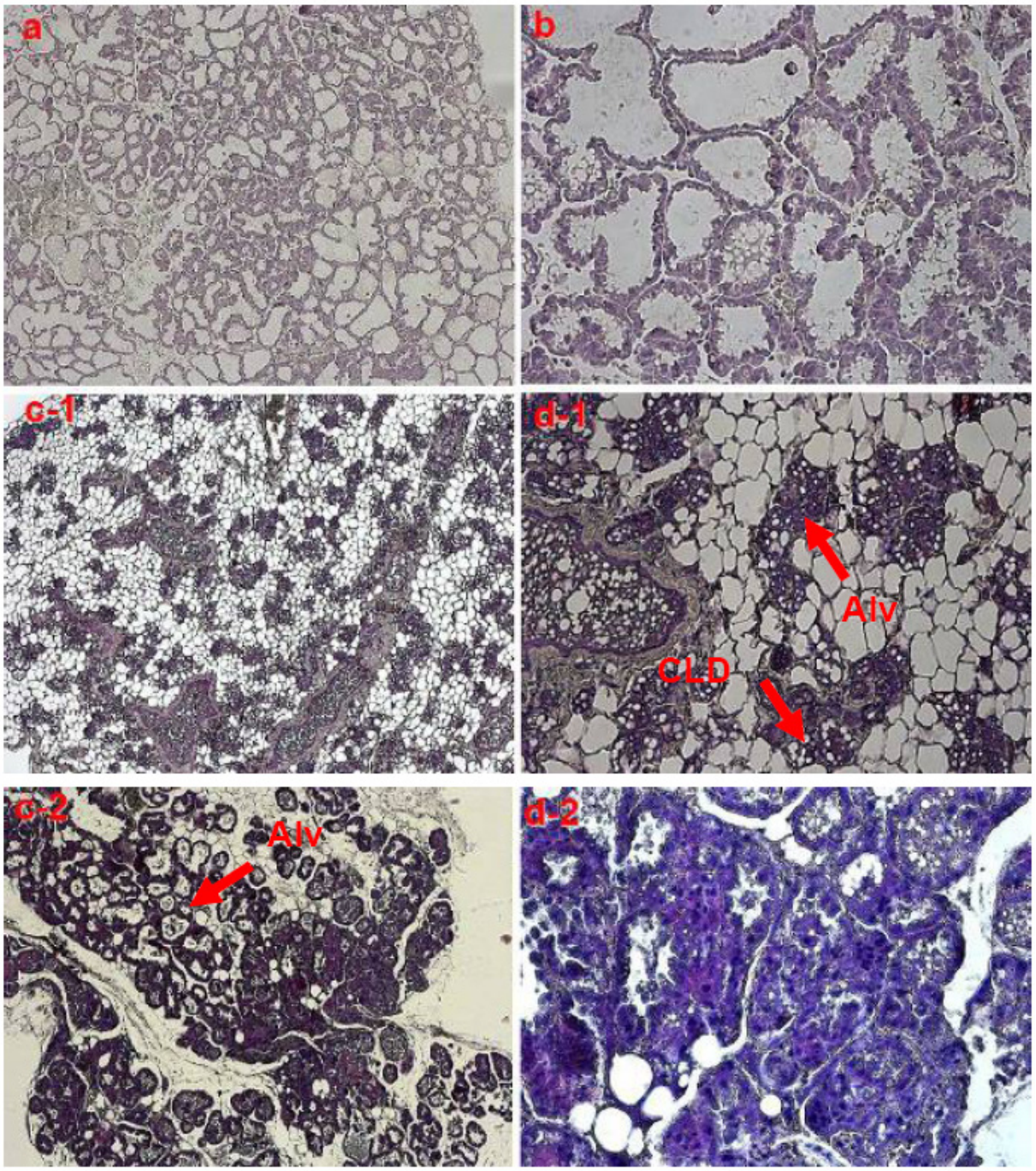


Figure 31: Histological features of the mammary gland at the lactation day 14. All tissue sections were stained with HE. Wild type: a; ET-1: c1, c2 (x 50 magnification), wild type: b; ET-1: d1, d2 (x 200 magnification). Wild type mammary glands exhibited fully expanded alveoli reflecting the highly active secretory capacity (a, b). ET-1 transgenic mammary glands exhibited two different histological architectures, some parts of the mammary gland represent precocious involution with collapsed alveoli and increased adipocytes. Some of the alveoli (Alv) are still carrying CLDs (c1 and d1) at lactation day 14 pointing to a secretory defect. On the other hand ET-1 transgenic mammary glands exhibited focal hyper proliferative lesions (c2, d2). Contrarily to the upper figures (c1, c2), alveoli appear normal in this part of the mammary gland. (c2).

#### 3.3.4.1 Immunohistochemisty

One indication of a neoplastic progression is the loss of normal tissue architecture, including polarity. Generally, primary breast carcinomas show a dramatic increase in the ratio of luminal to myoepithelial cells, and many invasive breast carcinomas essentially lack myoepithelial cells completely (Gusterson, et al., 2005) (Gudjonsson, et al., 2002).

As a basket-like network the myoepithelial cells are positioned between stroma and alveolar lumen. The myoepithelial cells are responsible for the movement of milk out of the alveoli. This process is accomplished by the contraction of myoepithelial cells. The place where the myoepithelium is located, serve to control many aspects of luminal function. It could regulate polarity, electrolyte and fluid flow, conduct the process of endocrine or paracrine signals (Lakhani; O'Hare, 2001).

An immunohistochemical method for the detection of myoepithelial and epithelial cells was employed to distinguish the normal mammary and neoplastic mammary gland (Gusterson, et al., 1982), (Gusterson, et al., 2005). Indeed, routine histopathological evaluations of mammary glands use the retention of the myoepithelial layer as a critical diagnostic criterion to discriminate carcinomas (Jones, et al., 2004). Therefore, immuno histostaining of smooth muscle actin (SMA) was employed to rule out an underlying malignant transformation.

Hematoxylin/eosin and SMA immunohistostaining was performed on parallel sections obtained from wild type and ET-1 transgenic mammary glands. The slides were immunostained with an anti-SMA antibody and show brown coloured myoepithelial cells enclosing the alveolar epithelial cells (blue colour, Figure 32,b1). The slides not treated with SMA antibody were utilized as negative control, ensuring specifity of the SMA immunohistostaining (Figure 32, a-2, b-2, c2, d-2). SMA immunostaining clearly manifests the non neoplastic structure by the presence of myoepithelial cells surrounding alveoli in the ET-1 transgenic females.

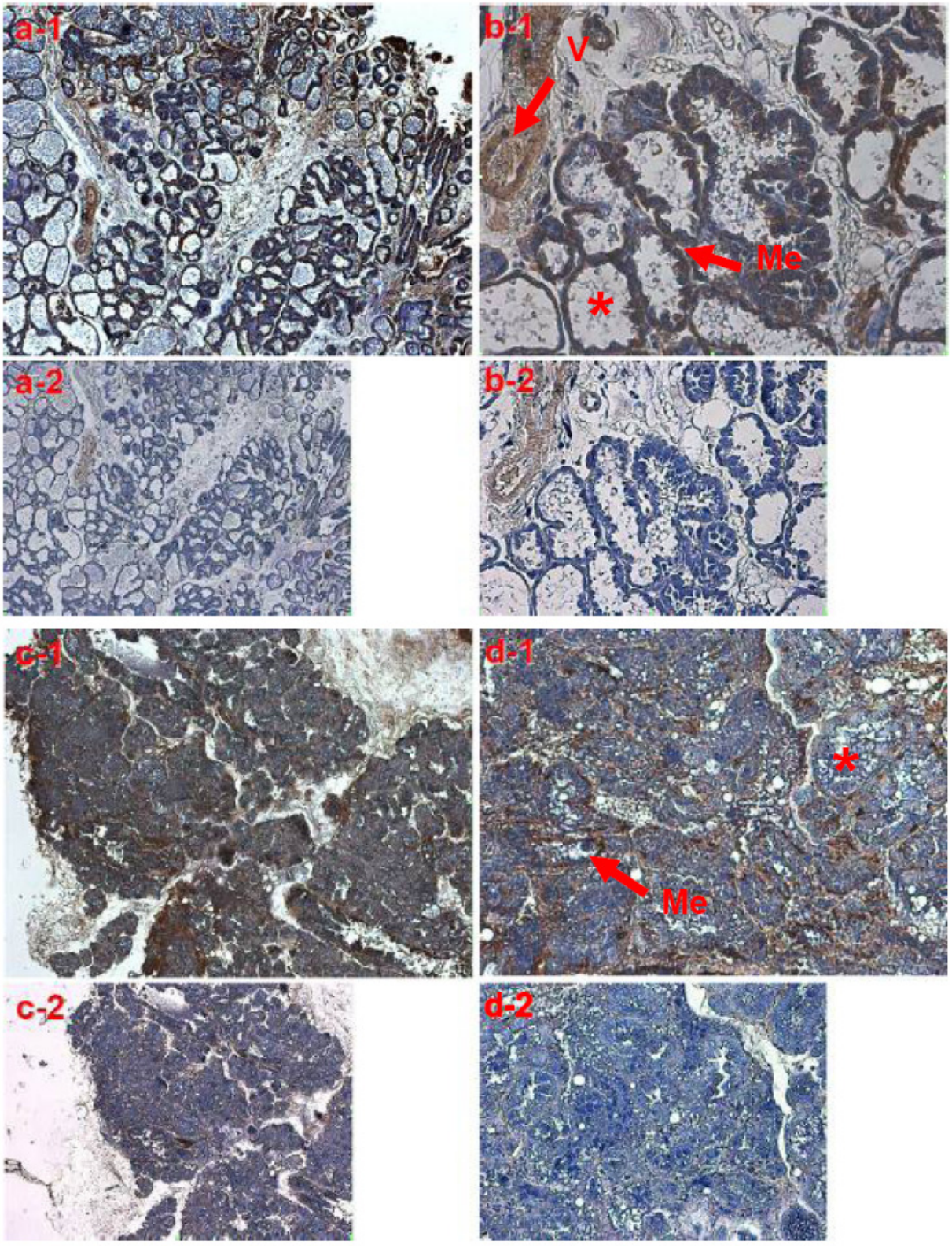


*Figure 32: Analysis of SMA immunohistostaining in wild type and ET-1 transgenic mammary glands at 14th day of lactation. Wild type: a-1, a-2; ET-1: c-1, c-2 (x 50 magnification), wild type: b-1, b-2; ET-1: d-1, d-2 (x 200 magnification). Myoepithelium (Me) was stained by a SMA specific immunohistostaining method; Brown coloured areas in both groups indicated the SMA. In order to visualize any possible cross reactivity for SMA staining parallel slides were treated without SMA antibody as negative control a-2, b-2, c-2, d-2. The wild type group demonstrated well organized alveoli (*) and the individual alveoli are surrounded by blood vessels (V). On the other hand the ET-1 group demonstrated hyper proliferative epithelium with SMA staining alveolar lumen and borders of alveoli (*) become visual.*

In ET-1 transgenic mammary glands, markedly enlarged lobules are composed of epithelial cells featuring a vacuolated cytoplasm and nuclei with prominent nucleoli. SMA immunostaining clearly demonstrated that ET-1 transgenic mammary glands have non neoplastic tissue architecture with the presence of myoepithelial cells surrounding these alveoli. Following the lactation period, ET-1 transgenic mice were analysed for the presence of benign tumours at the involution day 14. Throughout the involution no proliferative lesions were detected (Figure 33).

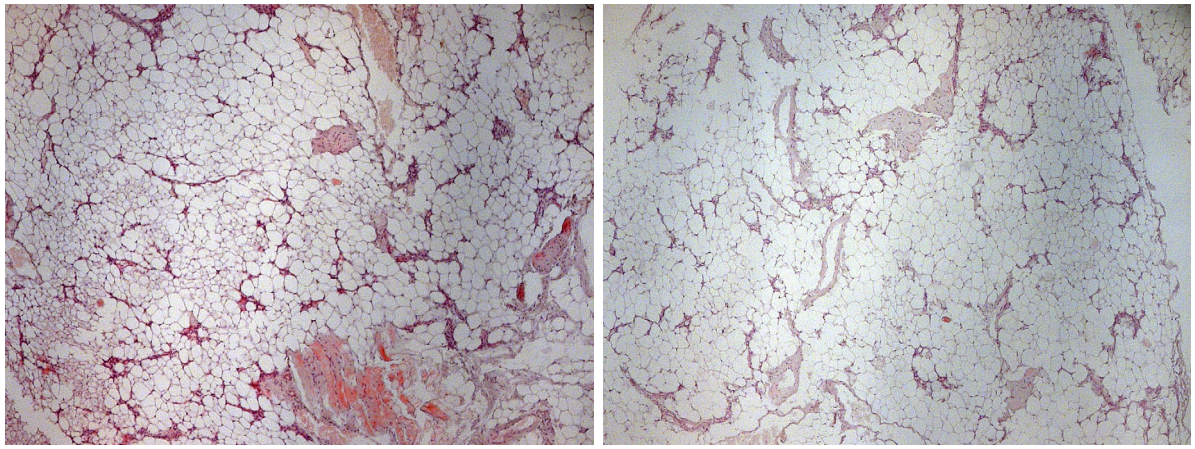

*Figure 33: Histological features of the mammary gland at the 14$^{th}$ day of involution. The tissue sections were stained with hematoxylin and eosin of wild type (left) and ET-1 transgenic mammary glands (right) (x 50 magnification). Both mammary glands showed same histological patterns of involution with the adipocytes and rudimentary epithelium. No hyperproliferative lesion or any type of irregularity was detected in both animal groups.*

Taken together, these findings are indicative of lactational hyperplasia, a reversible and benign breast lesion found only in pregnancy and lactation (Jones, et al., 2004), (Sabate, et al., 2007). The morphological analysis of the mammary glands in ET-1 transgenic mice revealed the following key findings: No morphological changes exhibited in virgin and pregnant animals. Following the parturition, the presence of CLDs in alveoli and the reduced alveolar diameters are indicating a defect on secretion or postpartum proliferation. Furthermore, the structural changes at lactation day 3 are pointing to precocious involution and, in addition, at lactation day 14, the ET-1 transgenic mice exhibited lactational hyperplasia. Therefore, the analyses of the mammary glands concentrated on the biochemical and molecular characterisation of these histological changes.

## 3.4 Molecular characterization of the secretory activation in ET-1 transgenic animals

The secretory activation occurs after parturition and converts the inactive lobuloalveoli to active milk secretion. This process is controlled by a drop in the level of progesterone and an augmented prolactin signalling (Anderson, et al., 2007), (Naylor, et al., 2005). These hormo-

nal changes are characterized by the activation of a number of functionally important genes (Rudolph, et al., 2007) in order to maintain milk production and promote secretory activation of the mammary gland.

At pregnancy day 18, the histological analysis of the wild type mice and ET-1 transgenic mammary glands revealed no differences. However at the lactation day 3, striking differences had been exposed. Therefore, the following experiments were concentrated by employing real time PCR to evaluate the expression of genes known to be involved in secretory activation and milk production

### 3.4.1 Expression of milk proteins

Lactation is defined as the copious production of milk. By gaining secretory capacity, the expression of milk protein genes is increased in order to support the growth of the newborn. (Neville, et al., 2002). Therefore, monitoring the expression of milk proteins could reflect the functional differentiation of the mammary gland on the molecular level. In this sense the most prominent milk proteins, WAP, Beta-casein and Alpha-lactalbumin gene expression were analysed by real time PCR.

A significant downregulation of WAP (to about 10%) and Beta-casein (to about 50%) could be detected in ET-1 transgenic mammary glands (Figure 34), demonstrating a lactational incompetence on the molecular level. However, Alpha-lactalbumin levels remained unchanged (Figure 34).

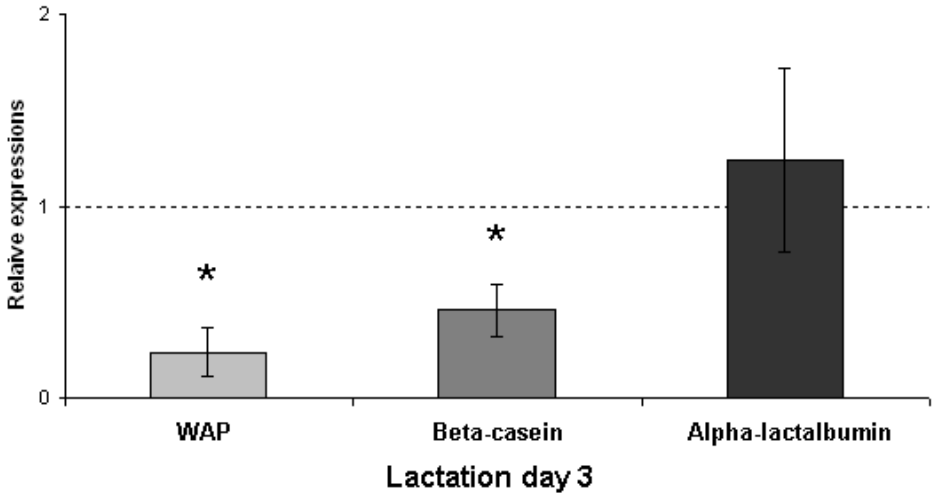


*Figure 34: Analysis of WAP, Beta-casein and Alpha-lactalbumin expression at lactation day 3. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002) P<0,05 n=5 mice for each group.*

Gene expression of milk proteins is coordinated in a well defined pattern, whereby Beta-casein is expressed during early pregnancy. In contrast, WAP and Alpha-lactalbumin ex-

pression is initiated only late in pregnancy (Robinson, et al., 1995), (Naylor, et al., 2005). On lactation day 3 a significant downregulation of WAP and Beta-casein gene expression was detected. Therefore these down regulated genes were analyzed at pregnancy day 18. Similar to lactation day 3 expression of WAP was found to be significantly down regulated in late pregnancy compared to wild type mammary glands. However, Beta-casein RNA expression level was not different at pregnancy day 18 from the wild type counterpart (Figure 35).

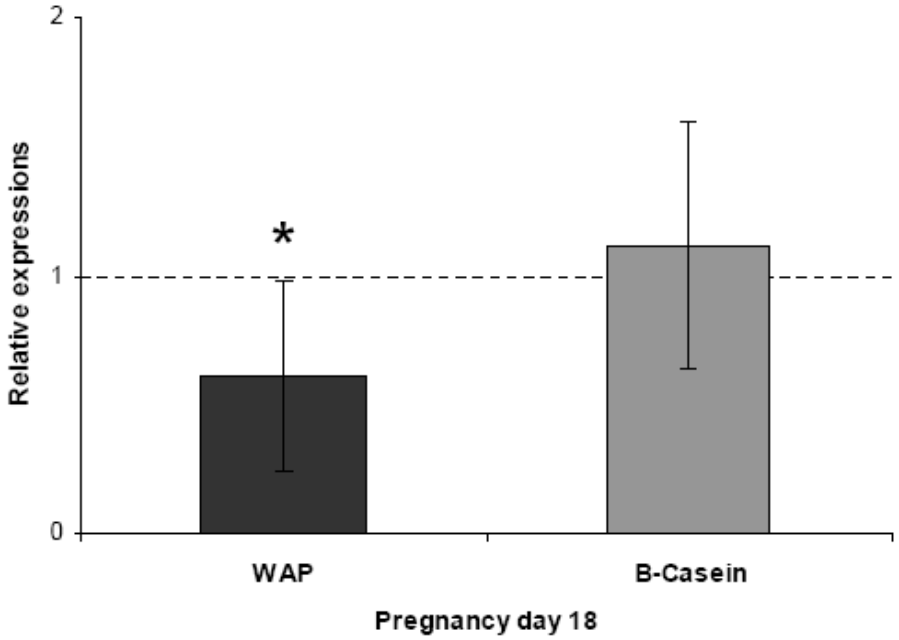


*Figure 35: Analysis of WAP and Beta-casein expression at pregnancy day 18. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002) P<0,05 n=5 mice for each group.*

### 3.4.2 Expression of other secretory activation markers

Milk production and secretion is mediated by a diverse and complex array of transport and secretory processes that are regulated by developmental and physiological factors. A possible damage in the regulation of tight junction proteins or synthesis and transport processes of glucose and lipids in the mammary gland can lead rapidly to a phenotype (Neville, et al., 1990). It's known that the substantial regulation of these processes occurs at the level of mRNA expression (Rudolph, et al., 2007)). Therefore, to further characterize the assumed developmental defects in mammary glands of ET-1 transgenic mice, Claudin-8, GLUT-1 and SREBF-1 mRNA expression levels were studied by real time PCR on day 3 of the lactation period, since these genes are known to play an important role in lactation and lipid synthesis in the mammary gland (Blackman, et al., 2005), (Seagroves, et al., 2003), (Camps, et al., 1994), (Rudolph, et al., 2007).

Whereas the mRNA expression levels of Claudin-8 and GLUT-1 remained unchanged, SREBF-1 expression was significantly up-regulated in ET-1 transgenic mammary glands in

comparison to wild type animals (Figure 36). SREBF-1 is a member of a transcription factor family which is recognized as regulating fatty acid and cholesterol biosynthesis. It has been noted that SREBF-1 is a critical regulator of secretory activation regarding to lipid biosynthesis, in response to AKT activation (McManaman; Neville, 2003).

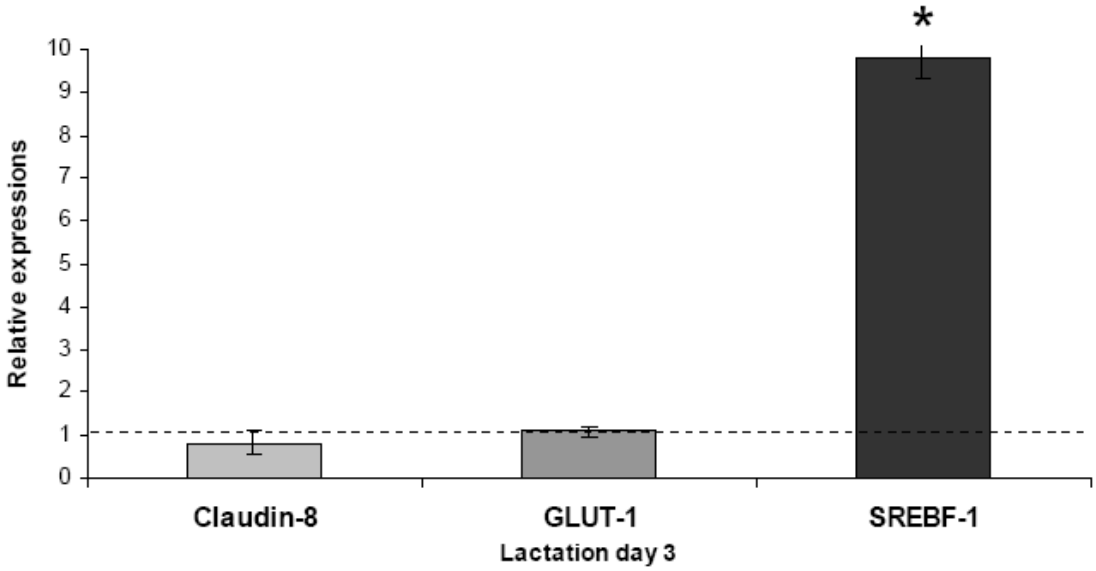


*Figure 36: Analysis of the expression of claudin-8, GLUT-1 and SREBF-1 from the transgenic glands in ET-1 transgenic group compared to wild type group at lactation day 3. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002)P<0,05 n=5 mice for each group*.

To further determine the effect of the ET-1 overexpression on the upregulation of SREBF-1, pregnancy day 18 was also analyzed. However, SREBF-1 mRNA expression did not differ when compared to wild type at pregnancy day 18 (Figure 37).

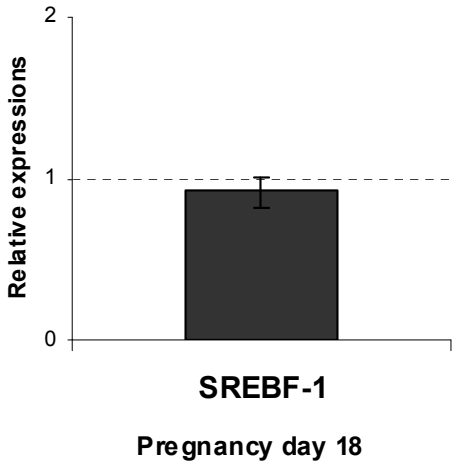


*Figure 37: Analysis of the expression of SREBF-1 in ET-1 transgenic mice at pregnancy day 18. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002), n=5 mice for each group no significant difference was observed.*

### 3.4.3 STAT5 activation

Among the factors which influences the mammary gland development is the transcription factor STAT5 which is essential for prolactin induced terminal differentiation of mouse mammary epithelial cells during pregnancy and lactation (Naylor, et al., 2005). Binding of prolactin to its receptor activates STAT5 which in turn controls the alveolar expansion as well as WAP gene expression (Liu, et al., 1997). Therefore, activity of STAT5 in wild type and ET-1 mice were compared to identify whether STAT5 signaling contributes to the downregulation of WAP and histological alteration in ET-1 transgenic mice.

The STAT5 activity is monitored by the relative phosphorylation level of the STAT5 protein using immunoblot techniques and densitometric analyses. However, no significant differences could be found in the activation of STAT5, neither at pregnancy day 18 nor at lactation day 3 (Figure 38, 39).

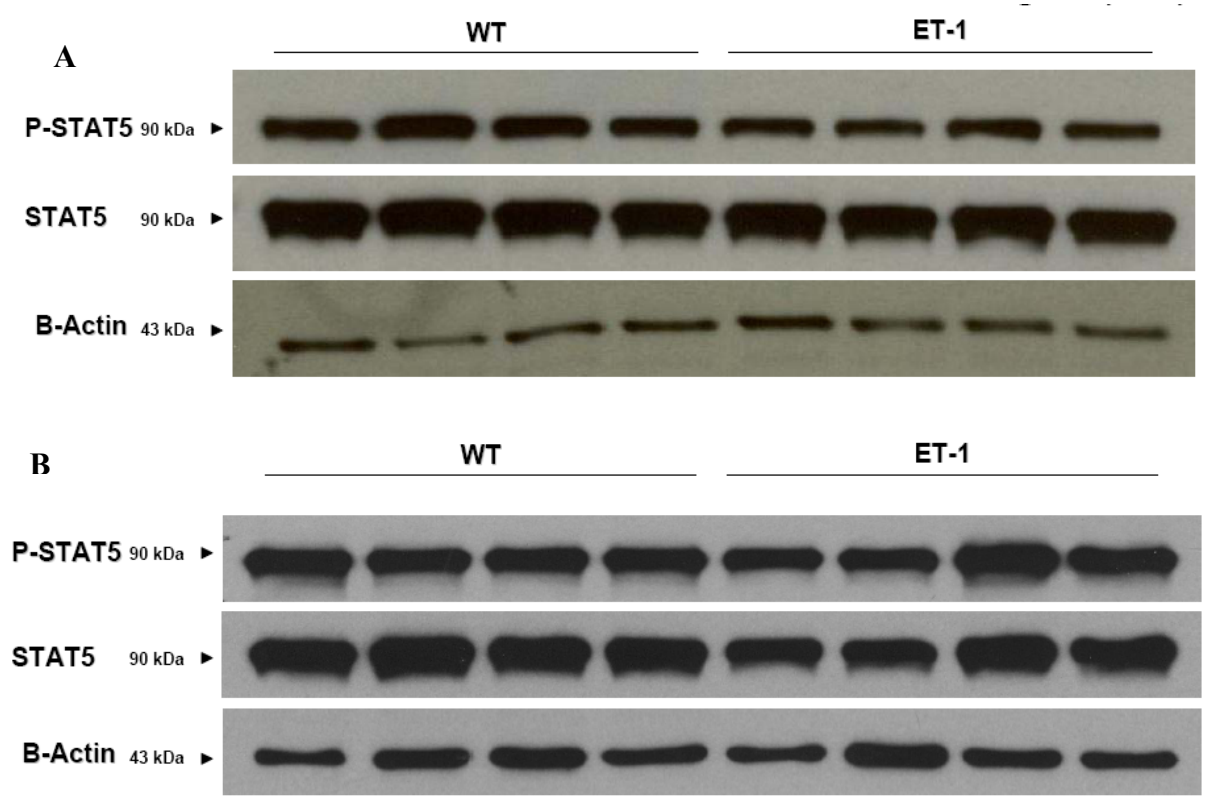


*Figure 38: Protein expression and phosphorylation of STAT5 protein were determined by western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. A phosphospecific (Y634) STAT5 antibody and a STAT5 antibody were used to monitor activated STAT5. Blot A: 18$^{th}$ day of pregnancy, blot B: 3$^{rd}$ day of lactation.*

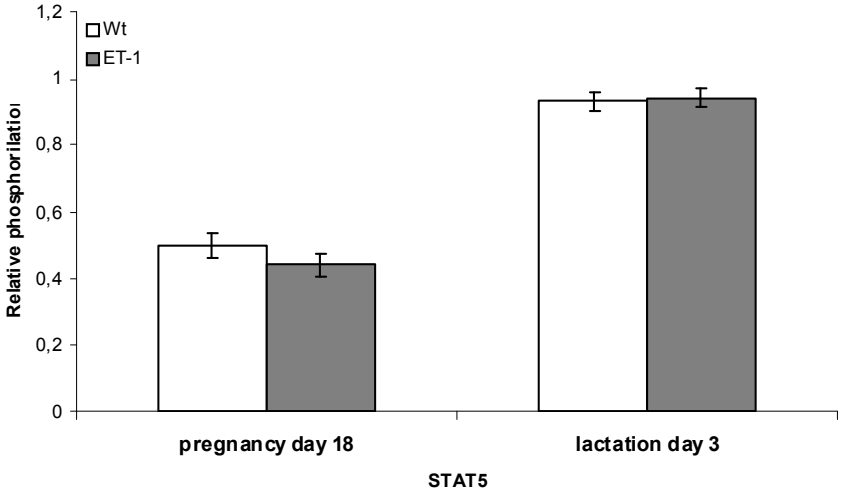


*Figure 39: A representative densitometric analysis of relative STAT5 phosphorylation in wild type and ET-1 transgenic mice at 18th day of pregnancy and at 3rd day of periods shows no statistical significance for ET-1 transgenic mice in comparison to wild type counterparts (n=4) females for each group no significant difference was observed.*

### 3.4.4 AKT activation

AKT is involved in the functional differentiation of the secretory epithelium during pregnancy (Maroulakou, et al., 2008) and plays a role in the metabolic pathways that regulate milk synthesis during lactation (Schwertfeger, et al., 2001). It has been noted that increased AKT activity resulted in increased lipid biosynthesis and glucose transport (Schwertfeger, et al., 2001). In addition to these functional and metabolic importances, AKT acts as a survival factor for secretory epithelial cells. Accordingly, its expression decreases rapidly with the onset of involution (Maroulakou, et al., 2008).

Therefore, the analysis of AKT activity and protein expression in wild type and ET-1 transgenic mice might highlight some of the molecular differences being at work in these two groups. As shown in Figures 40 and 41 there is no statistical difference between the two groups in relative phosphorylation status of AKT in each developmental stage of the mammary gland.

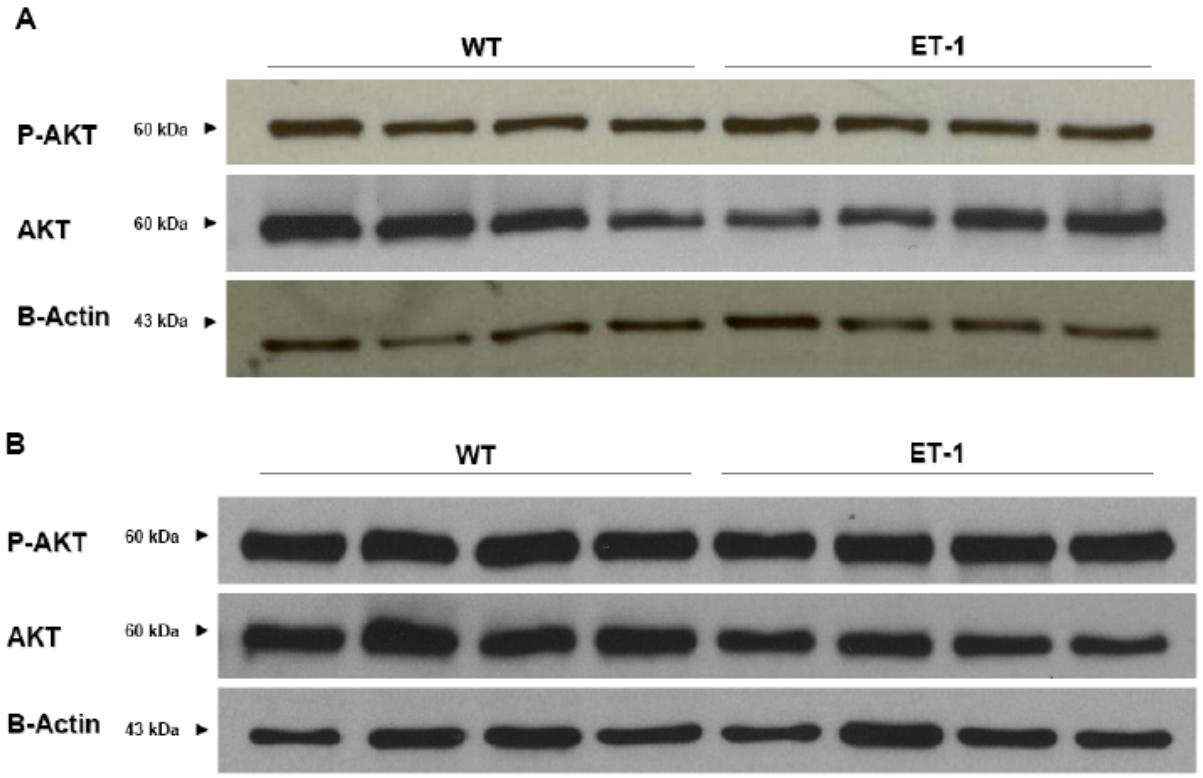


Figure 40: Protein expression and phosphorylation of AKT protein were determined by western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. A phosphospecific (Y334) AKT antibody and AKT antibody were used to monitor activated AKT. Blot A: 18$^{th}$ day of pregnancy, blot B: 3$^{rd}$ day of lactation.

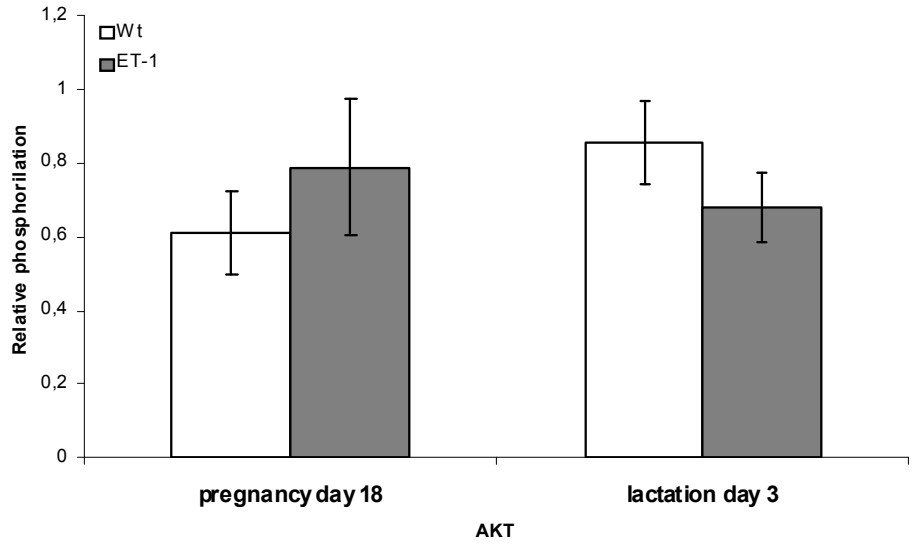


*Figure 41: A representative densitometric analysis of relative AKT phosphorylation in wild type and ET-1 transgenic mice at 18$^{th}$ day of pregnancy and at 3$^{rd}$ day of lactation periods shows no statistical significance for ET-1 transgenic mice in comparison to wild type counterparts (n=4 for each group) .*

### 3.4.4.1 AKT protein expression

As mentioned before, AKT acts as a survival factor for secretory epithelial cells Therefore AKT is critical for the suppression of involution during lactation (Maroulakou, et al., 2008).

In addition to the analysis of AKT activation, the protein expression level of AKT was also analyzed. Figure 42 shows a densitometric analysis of AKT on the protein expression during lactation day 3. ET-1 transgenic mice exhibited a significant decrease of AKT on the protein level compared to the wild type group.

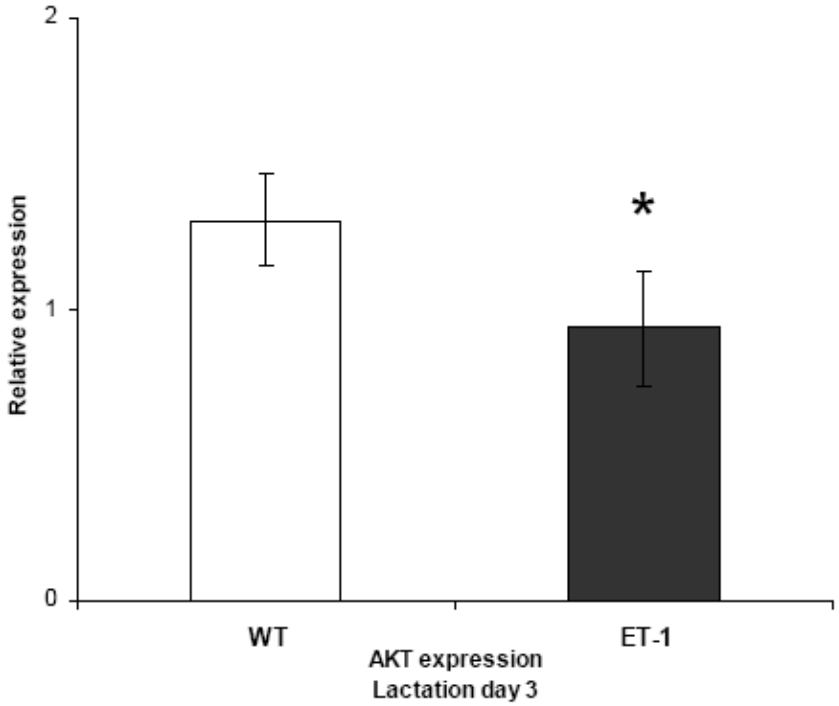


*Figure 42: A representative densitometric analysis of the protein expression of AKT in wild type and ET-1 transgenic mice at the 3rd day of lactation shows a significant downregulation in ET-1 transgenic mice in comparison to wild type counterparts (n=4 mice for each group; (*) P<0.05 Student´s T-test).*

## 3.5 Molecular characterization of precocious involution

Mammary gland involution is defined as a transition of the lactating gland to the non-lactating stage in preparation for a subsequent pregnancy. The involution is a two steps process according to its reversibility (Lund, et al., 2000). Generally, the involution process elicits after cessation of milking, sudden weaning of the offspring or gradual decrease in suckling. During involution, the milk producing lobuloalveolar structures regress to a simple ductal tree.

In addition to an increased amount of adipocytes and focal collapsed alveoli in ET-1 mammary gland histology, a decrease of AKT protein expression is supporting evidence of a precocious involution at lactation day 3. However, involution is a multistep process which is controlled by many factors and genes. Therefore STAT3 activation is analyzed as a critical

molecule for the initiation of involution. In parallel, to the STAT 3 activation LIF and IL6 genes were also analyzed for the molecular validation of the observed involution process in ET-1 transgenic mice at lactation day 3.

### 3.5.1 STAT3 activation

STAT3 signaling is a key mediator of apoptosis during mammary gland involution (Kritikou, et al., 2003). The histological features detected in the ET-1 transgenic mammary glands exhibited signs of precocious involution, suggesting an increase of STAT3 activity in ET-1 transgenic glands.

Therefore, the relative phosphorylation of STAT3 was analyzed in both, pregnancy and lactation periods. As shown in Figure 43 A at pregnancy day 18 STAT3 is inactive in both groups. As expected, Figure 43B demonstrates STAT3 signaling activity at the onset of lactation in ET-1 transgenic mice. Densitometric analysis showed that this increase in activity is statistically significant (Figure 44).

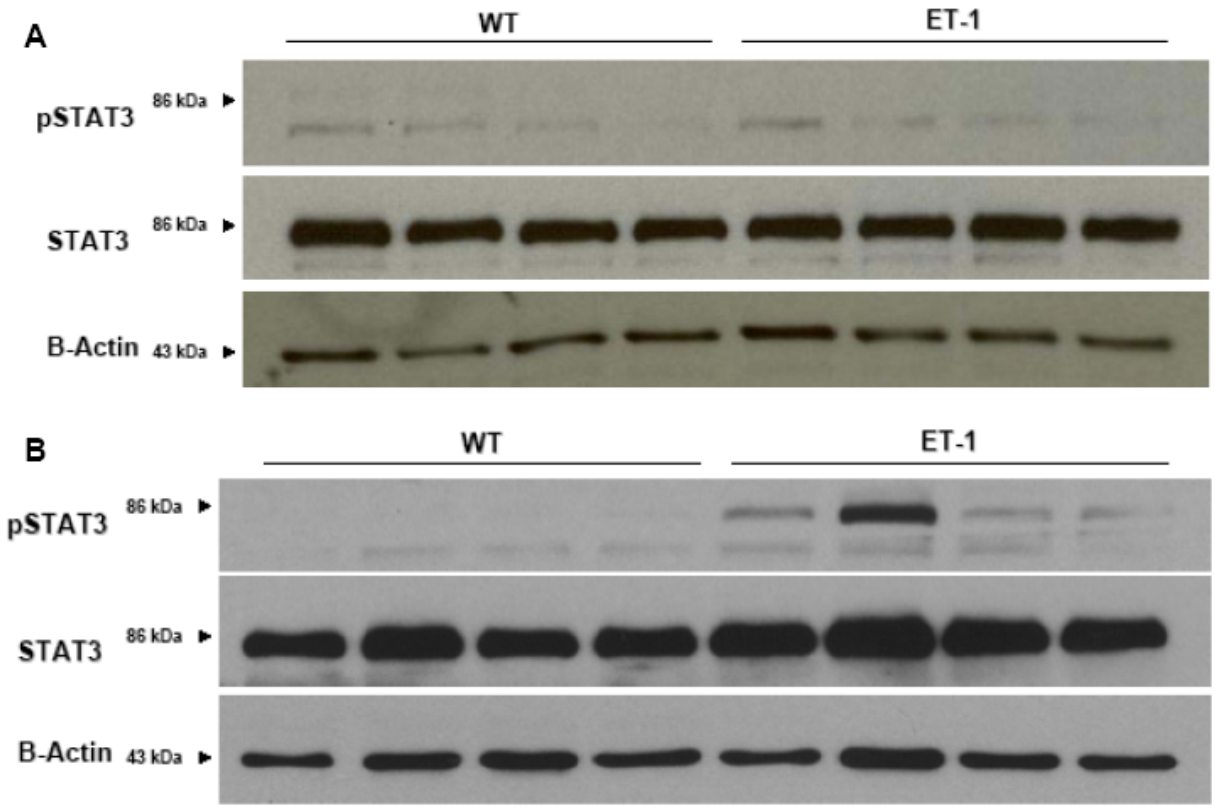


*Figure 43: Protein expression and phosphorylation of STAT3 protein was determined by western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. A phosphospecific (Y705) STAT3 antibody and a STAT3 antibody has been used to monitor activated STAT3. Blot A: 18$^{th}$ day of pregnancy, blot B: 3$^{rd}$ day of lactation.*

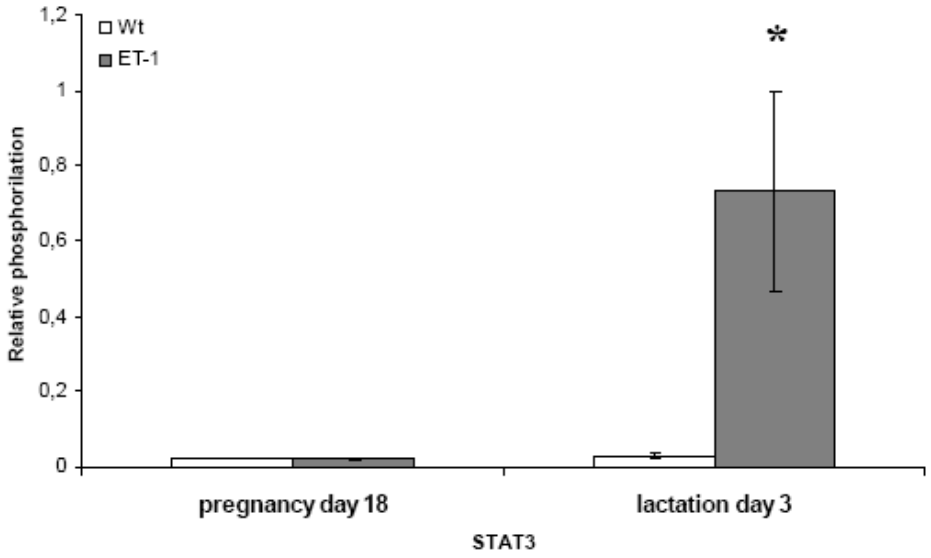


*Figure 44: A representative densitometric analysis of relative STAT3 phosphorylation in wild type and ET-1 transgenic mice at 18th day of pregnancy and at 3rd day of lactation periods shows a significant increase in ET-1 transgenic mice in comparison to wild type counterparts at lactation day 3 (n=4 mice for each group; (*) P<0.05 Student´s T-test).*

### 3.5.2 Expression of involution markers

It has been reported that a huge number of factors could trigger the involution process. Furthermore, over 500 transcripts are dramatically upregulated in the first 12 h of involution and declining immediately thereafter (Clarkson, et al., 2004). Therefore, it was of importance to analyse molecules known to be involved in the initiation of involution via the STAT3 pathway.

IL-6 and LIF are cytokines that are involved in the activation of STAT3 in various tissues (Akira, et al., 1994) and STAT3 activation was reported to play a crucial role in involution (Kritikou, et al., 2003). Therefore, expression of these genes was assessed using quantitative real time PCR. IL-6 expression in ET-1 transgenic mammary glands compared to wild type glands remained unchanged, whereas LIF expression was found to be significantly upregulated not only at lactation day 3 (Figure 45) but also at pregnancy day 18 (Figure 46) in the ET-1 transgenic mice.

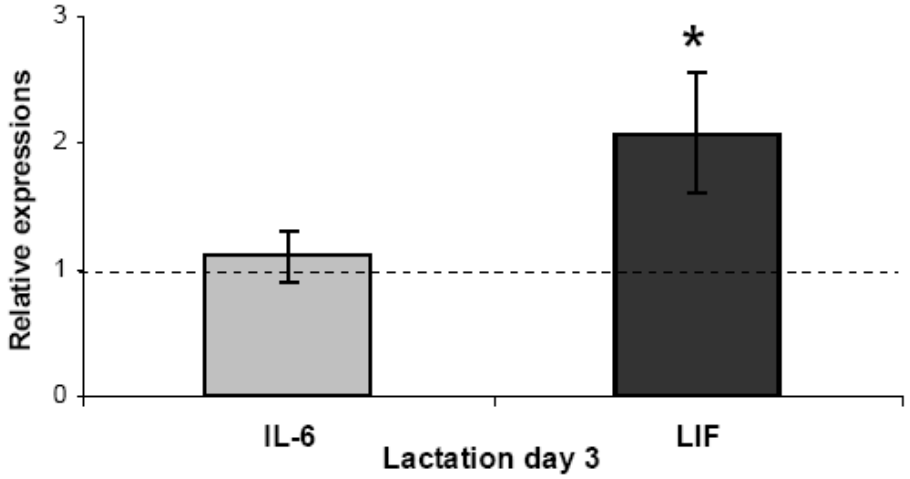


*Figure 45: Analysis of the relative expression of IL-6 and LIF at lactation day 3. The relative expression levels were compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002). P<0,05 n=5 mice for each group.*

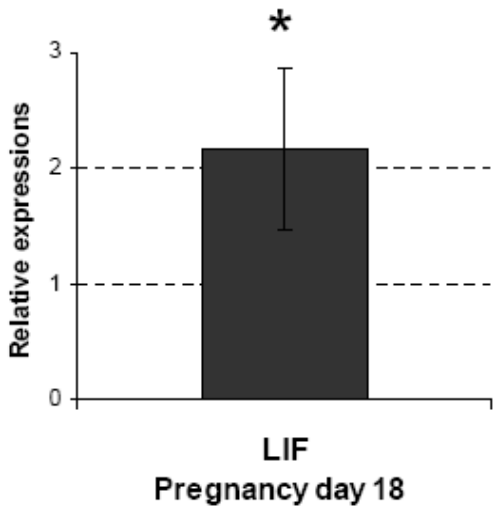


*Figure 46: Analysis of the relative expression of LIF at pregnancy day 18. The relative expression level was compared to wild type mice expression level, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002). P<0,05 n=5 mice for each group.*

## 3.6 Molecular characterization of lactational hyperplasia

ET-1 transgenic mice displayed focal and intense lobular proliferation in various grades. This unusual histological formation was characterised by markedly enlarged lobules with increased cytoplasmic volume and enlarged nuclei. Further, alveolar organization was lost in parts of these areas. These histological observations pointed to focal lactational hyperplasia in ET-1 transgenic mammary glands during the middle of the lactation period. A possible causative effect of ET-1 overexpression on the development of lactational hyperplasia might have been mediated by the interaction of its GPCRs with the EGFRs, since ET-1 is known to possess growth regulatory properties (Battistini, et al., 1993); (Cazaubon, et al., 1994). Therefore, to further characterize the assumed signaling network in ET-1 transgenic mam-

mary glands, EGFR activation and expression of its major ligands (EGF, TGFa, HB-EGF and amphiregulin) were analyzed at lactation day 14.

As described before ET-1 transgenic mammary glands exhibited precocious involution during early lactation. However, ET-1 transgenic mothers continued to serve milk to their pups. This observation is suggesting a postponement of on going involution. It has been noted that increased ERK activity could suppress the apoptosis (Iwasawa, et al., 2009). Moreover, the ERK signaling pathway is a key down stream pathway molecule for ET-1 induced EGFR transactivation (Cramer, et al., 2001) which might be related to lactational hyperplasia and postponement of involution. Therefore, activities of ERKs were analysed not only at lactation day 3 but also at pregnancy day 18 in order to demonstrate the effect of ET-1 overexpression on ERK activation in vivo situations.

### 3.6.1 EGFR activation

EGFR activation has an important role in mammary ductal outgrowth and branching (Fowler, et al., 1995), (Xie, et al., 1997). Additionally deregulation of the activity of this receptor has a strong correlation with tumour progression (Tsujioka, et al., 2010). Lactational hyperplasia (see 3.4.4) could be related to EGFR activation due to its central role on tumourigenesis. Therefore EGFR phosphorylation was analyzed at lactation day 14 using immunoprecipitation techniques. As demonstrated in figures 47 and 48 an increased phosphorylation at residue Y845 of the EGFR was observed in ET-1 transgenic mice compared to wild type.

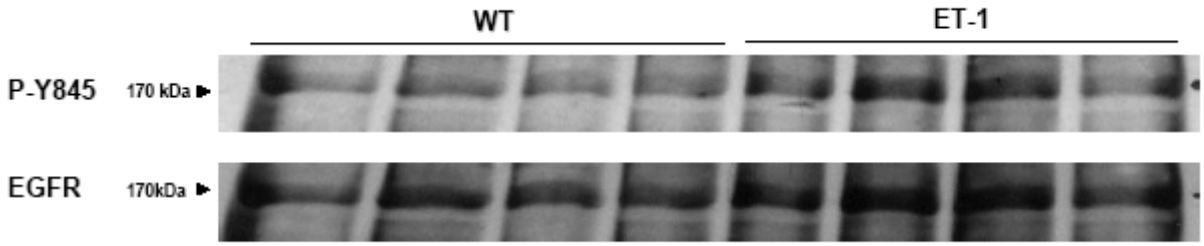


*Figure 47: Analysis of the EGFR phosphorylation at lactation day 14 was determined by immunoprecipitation. ~400 µg protein lysate for each sample has been used for immunoprecipitation with an EGFR specific antibody (withY1005), activation of EGFR has been monitored by phosphor specific antibody Y845 and Y1005 served as loading control.*

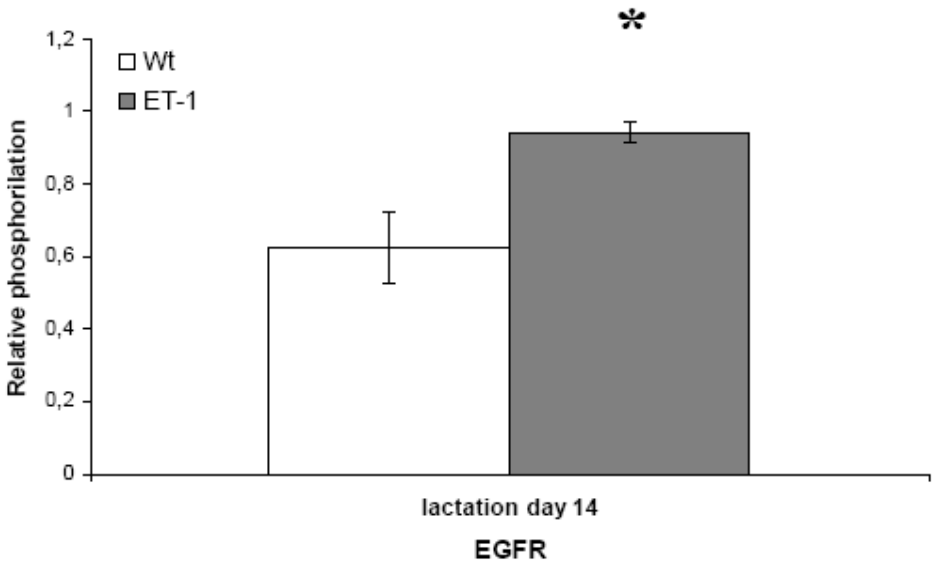


*Figure 48: A representative densitometric analysis of relative EGFR phosphorylation in wild type and ET-1 transgenic mice on the 14th day of lactation. ET-1 transgenic mammary glands exhibited significantly more phosphorylated EGFR than the wild type counterparts (n=4 mice for each group; (*) P<0.05 Student´s T-test).*

### 3.6.2 Expression of EGFR ligands

In order to identify ligands potentially involved in the observed activation of the EGFR, the expression of EGF, TGFa, HB-EGF and amphiregulin was quantified at the transcript level. As shown in figure 49, expression analyses on the 14th day of lactation demonstrated only a significant increase for the amphiregulin level in ET-1 transgenic compared to wild type mice, whereas the expression level of other ligands remained unchanged.

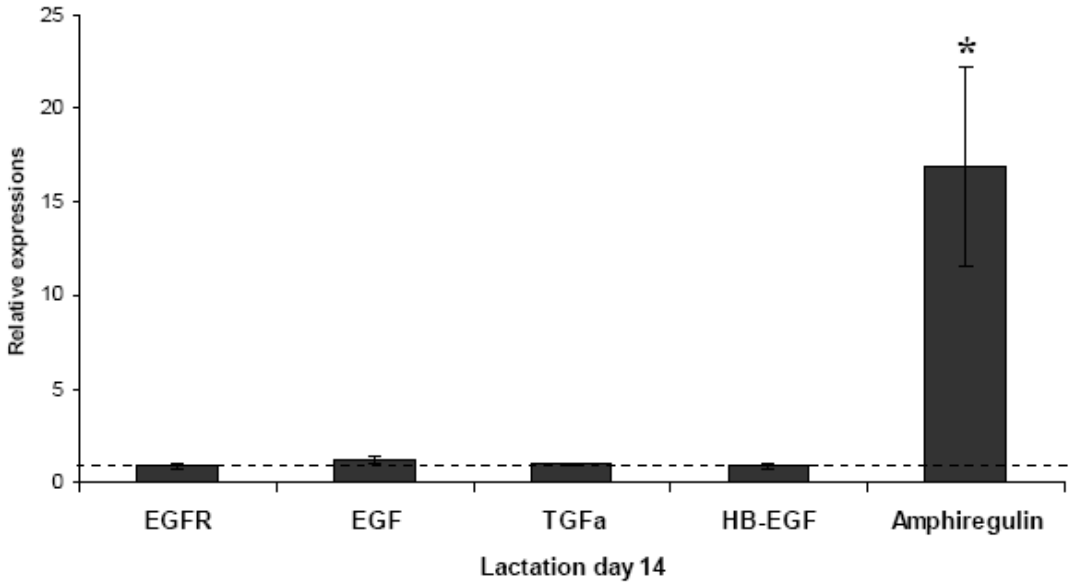


Figure 49: Relative expression of EGFR and its prominent ligands at lactation day 14. The relative expression levels were compared to wild type mice expression levels, which was set to 1 (dotted line). (*): Pair Wise Fixed Reallocation Randomisation Test (Pfaffl, et al., 2002) P<0,05 n=5 mice for each group.

Due to its functional role on ductal development of the mammary gland, amphiregulin expression was found to be increased during pregnancy but after parturition its transcription dramatically decreased (D'Cruz, et al., 2002), (Schroeder; Lee, 1998). To demonstrate the effect of ET-1 overexpression on amphiregulin expression, RNA levels of amphiregulin were analyzed at pregnancy day 18. As depicted in Figure 50 amphiregulin is also significantly upregulated at pregnancy day 18.

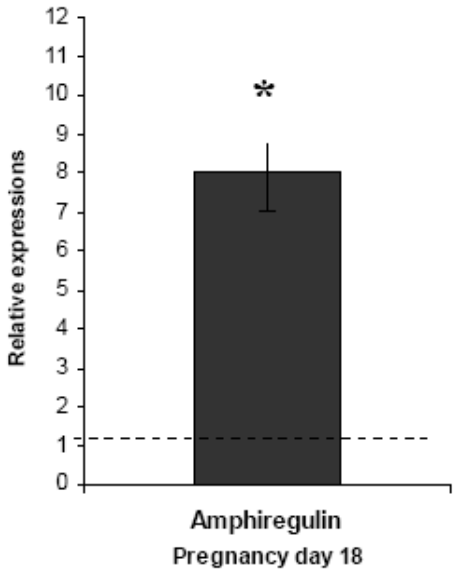


*Figure 50: Relative expression of amphiregulin at pregnancy day 18. The relative expression levels were compared to wild type mice expression levels, which was set to 1 (dotted line) (Pfaffl, et al., 2002) P<0,05, n=5 mice for each group*.

### 3.6.3 ERK activation

The most well defined signalling pathway from the cell membrane to ERK 1 and ERK 2 is that mediated by the EGFR (Hunter, et al., 1997), (Joslin, et al., 2007). ET-1 stimulates ERK 1 and ERK 2 through cognate GPCR receptors (Cramer, et al., 2001) by transactivation of the EGFR in various cell types and tissues (Hua, et al., 2003), (Kodama, et al., 2002). Additionally, increased ERK activation is documented in tubular hyperplasia as a result of ET-1 induction (Chu, et al., 2007). Therefore, ERKs represent key down stream molecules for ET-1 induced EGFR transactivation and might therefore be related to postponement of involution and initiation of lactational hyperplasia. Hence, ERK activation was analyzed at pregnancy day 18 and lactation day 3 (Figure 51; blot A and blot B and Figure 52). During pregnancy day 18, as well as during lactation day 3 a pronounced activation of ERK 1 and ERK2 signaling could be detected in the mammary glands of ET-1 transgenic mice when compared to wild type mice (Figure 51 and 52).

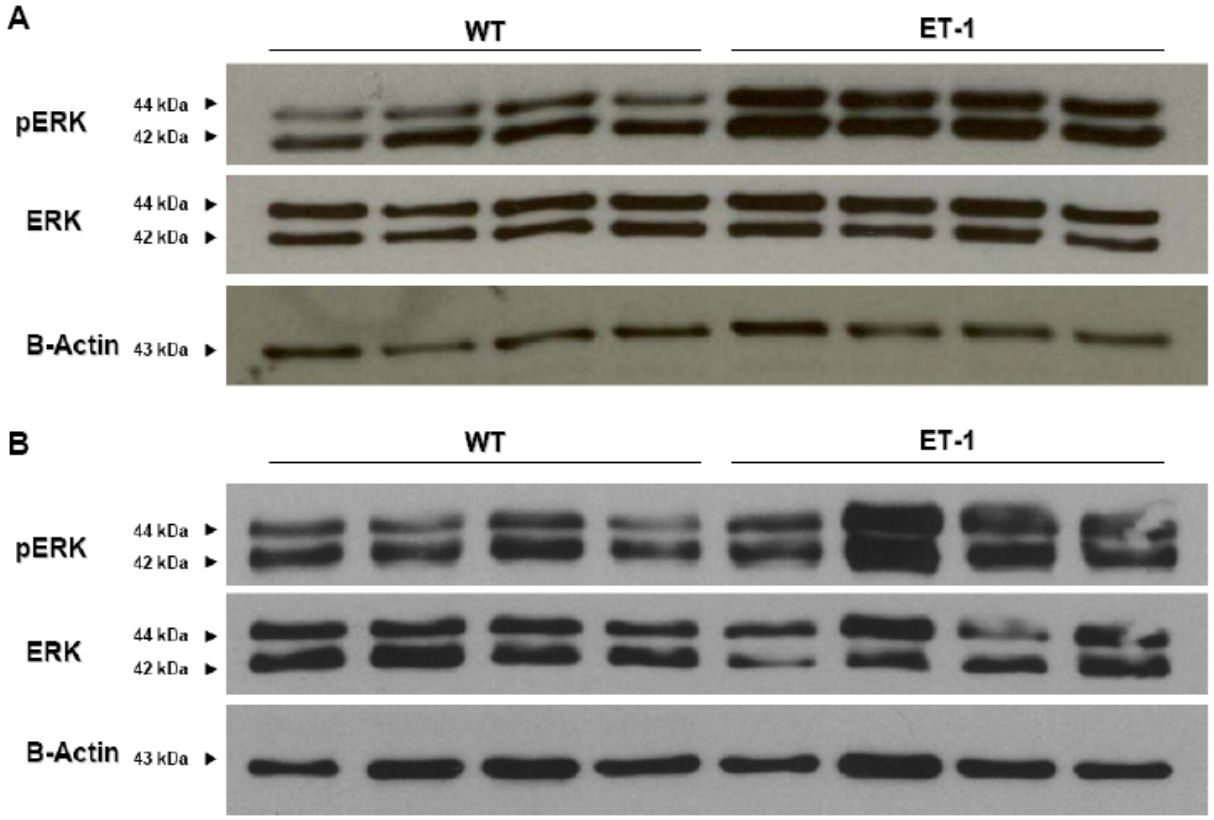


*Figure 51: Protein expression and phosphorylation of ERK 1 (44kDa) and ERK 2 (42 kDa) protein were determined by western blotting. Beta-actin served as control for loading. 25µg protein extract was loaded for each sample. A phosphospecific ERK1/2 antibody and an ERK1/2 antibody has been used to monitor activated ERK 1 and ERK 2. Blot A: 18th day of pregnancy, blot B: 3rd day of lactation.*

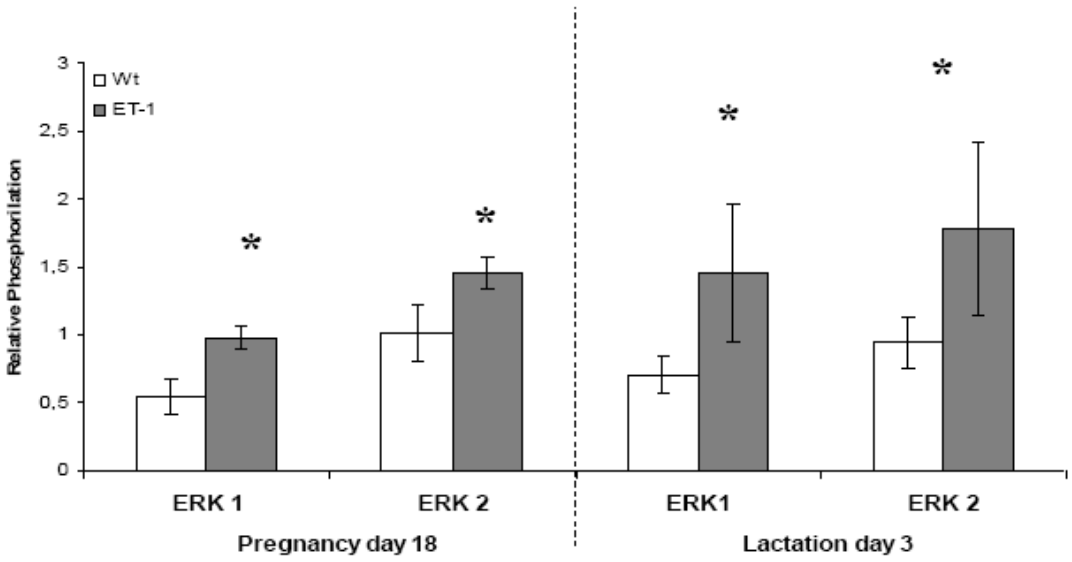


*Figure 52: A representative densitometric analysis of relative ERK1 and ERK2 phosphorylation in wild type and ET-1 transgenic mice at 18th day of pregnancy and at 3rd day of lactation. The ET-1 transgenic group exhibited significantly more phosphorylated ERK1 and ERK2 than wild type counterparts at both physiological stage (n=4 for each group; (*) P<0.05 Student´s T-test).*

## 3.7 Cell culture

ET-1 transgenic mice developed precocious involution and lactational hyperplasia during the lactation period. There is no lactating cell culture model available to confirm these in vivo results. However, cell culture studies could be considered as useful tools for the disclosure of possible cellular signalling pathways in response to ET-1 induction. Thus the mammary epithelial cell lines MCF7 and MDA MB 231 have been employed to verify some of the results which were obtained in in vivo experiments. As depicted in Figure 53 these cell lines have different endothelin receptor properties. MCF7 cells possess only ETAR receptors while MDA MB 231 cells possess both endothelin receptors, ETAR and ETBR, respectively.

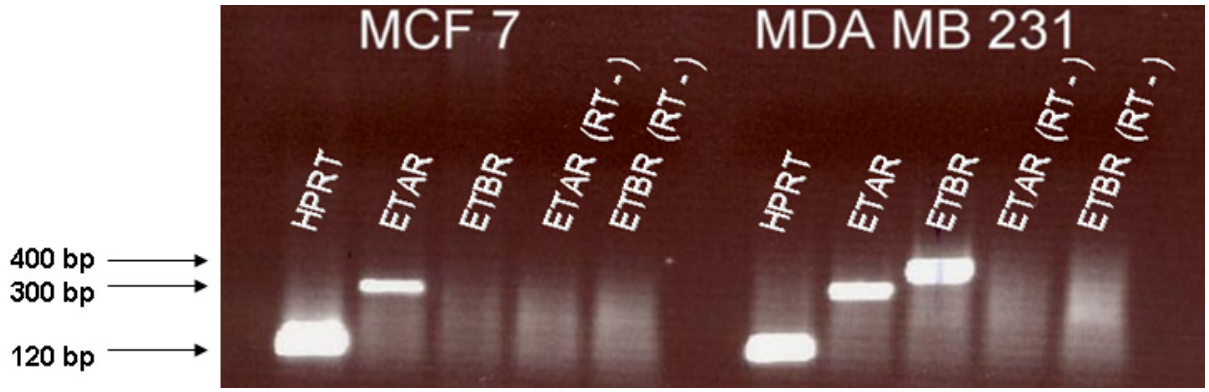


*Figure 53: Expression analysis of endothelin receptors in MCF7 and MDA MB 231 cells. MCF7 cells expressed only receptor A, on the other hand MDA MB 231 cells expressed both endothelin receptors. Any possible genomic DNA contamination in samples has been controlled by (RT-) PCR. HPRT, a housekeeping gene, was employed for loading control.*

### 3.7.1 cAMP assay

This assay technically measures cAMP which is produced in cells in response to the effect of an agonist such as ET-1. Active ET-1 has a very short half life of ca. 20 min (Levin, 1995). Its activity is affected by several factors, such as media composition, light, O2 and pH level. The cAMP test monitorizes in real time the response of the cells to ET-1 treatment. For all in vitro experiment, the concentration of ET-1 being used and its incubation time was according to (Boerner, et al., 2005). Cells were starved of serum overnight and left untreated or ET-1 treated ($10^{-6}$ M), for 10 min at 37°C. These concentrations of agonist were found to be optimal for the detection of the EGFR complex and phosphorylation of Y845 (Boerner, et al., 2005).

Forskolin is a known activator molecule of adenylate cyclase and is thus used as a positive control (Insel; Ostrom, 2003). Luminescence is inversely proportional to cAMP levels. Thus, the higher the cAMP concentration, the lower should be the luminescence measurement. In this sense ETAR should decrease the luminescence because of its $G\alpha_s$ activity. In contrast

ETBR should increase the luminescence because of its $G\alpha_i$ activity. (Takigawa, et al., 1995), (Goldsmith; Dhanasekaran, 2007)

A concentration of $10^{-6}$ M ET-1 was used to treat the cells. At this concentration, MCF7 cells responded with an increase in cAMP levels, whereas MDA MB 231 cells responded with decreased cAMP. This could be explained by the fact that MDA MB 231 cells express both endothelin receptors. Addition of ETAR blocker (BQ 123) to the medium enhanced the decrease of cAMP level. Addition of ETBR blocker (BQ 788) resulted in increased cAMP level. These data clearly proof that the ET-1 employed in these tests is active, as it can exert biological effects within the cells (Figure 54 and 55).

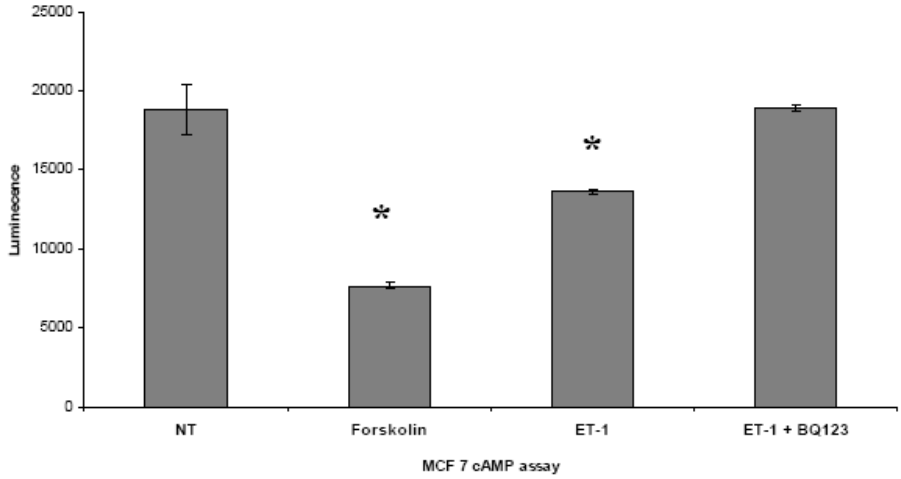


*Figure 54: Analyses of cAMP assay in MCF7 cells. Luminescence is inversely proportional to cAMP production. Forskolin was used as positive control for the cAMP assay, which increases the cAMP production. ET-1 treated cells produced significantly more cAMP than nontreated cells or a combination of ET-1 and the ETAR blocker BQ123. (n= 3 for each group; (*) P<0.05 Student´s T-test).*

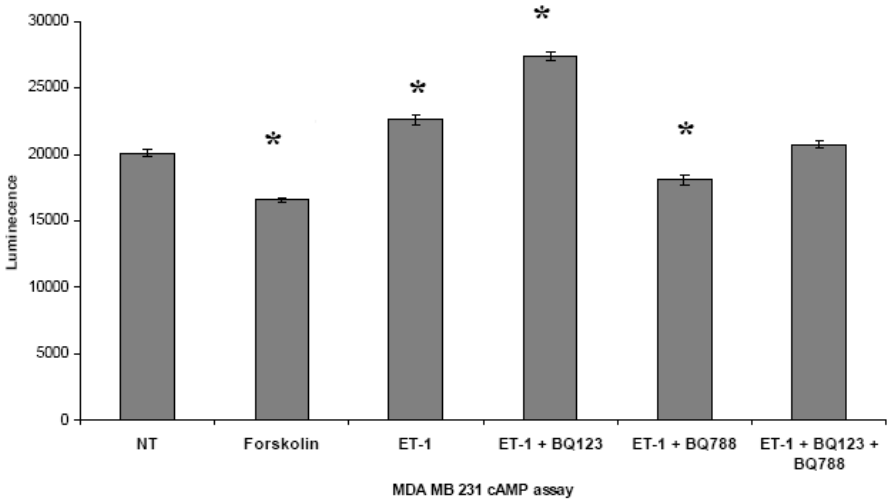


*Figure 55: Analyses of cAMP assay in MDA MB 231 cells. Luminescence is inversely proportional to cAMP. Forskolin was used as positive control for the cAMP assay which increases the cAMP production. ET-1 treated cells produced significantly less cAMP than nontreated cells or a combination of ET-1 receptor blockers (BQ123 or BQ788) and a coctail of its receptor blockers BQ123 + BQ788. In the digram the ETAR blocker provacated the decrease of cAMP level in MDA MB 231 cells on the other hand ETBR blocker increased the cAMP level (n= 3 for each group; (*) P<0.05 Student´s T-test).*

### 3.7.2 In vitro RNA analysis

WAP, LIF and amphiregulin genes in ET-1 transgenic mammary glands were differently expressed compared to wild type mammary glands. WAP, LIF and amphiregulin expressions were altered in both pregnancy and lactation periods, respectively, suggesting that ET-1 has links to the regulation of these genes. To determine, whether the observations made in transgenic animals could be reproduced in vitro, cells were treated with $10^{-6}$ M ET-1 and incubated at 37 °C for 1h. To analyse the expression of LIF and amphiregulin, real-time PCR was employed. Although the breast cancer cells originated from mammary epithelial cells, they do not express the WAP gene (Lubon; Hennighausen, 1987). Further, Nukumi and coworkers reported that WAP treatment in vitro reduced the progression of human breast cancer cells by inhibiting the proliferation (Nukumi, et al., 2007). Therefore WAP expression analysis was omitted from the in vitro studies. As shown in Figure 56, gene expressions did not differ between ET-1 treated and control cells in both, MCF7 and MDA MB 231cells.

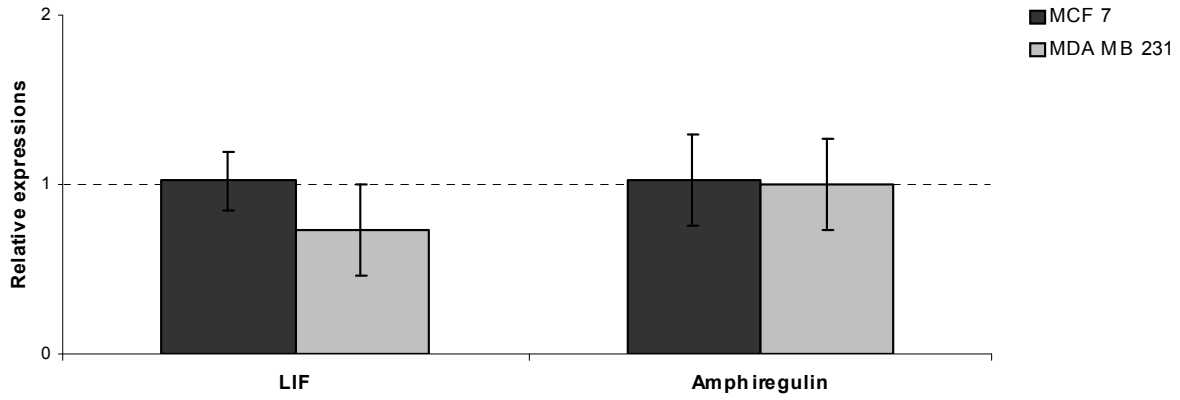


*Figure 56: Analyses of LIF and amphiregulin gene expression in vitro. The relative expression levels MCF 7 (dark grey) and MDA MB 231 (light grey) were compared to non treated cells which was set to 1 (dotted line). No significant difference was obtained in ET-1 treated cells.(Pair Wise Fixed Reallocation Randomisation Test has been performed for statistical analysis (Pfaffl, et al., 2002) no significant difference was observed n=5 for each group.*

### 3.7.3 Proliferation assays

In order to evaluate the functional effect of ET-1 on cellular behaviours such as proliferation or apoptosis in vitro, MCF-7 and MDA MB 231 cells were employed. The proliferation rate of the cell cultures were measured by employing two commercially available cell viability tests. The colorimetric MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), (Figure 57) and the colorimetric BrDU (5 bromdeoxyuridin) incorporation assay (Figure 58).

Cells were cultured in 96-well plates. After 24 h incubation, the medium was refreshed with FCS-free Dulbelco medium (DMEM) containing different concentrations of active human porcin ET-1 ($10^{-6}$ M, $10^{-8}$ M and $10^{-10}$ M) and the addition of 10% FCS served as positive control.

The cell cultures were treated with ET-1 for 24 h. After 24 h, colorimetric proliferation assays were performed.

As demonstrated Figure 57 and 58 respectively, none of these two proliferation assays could demonstrate such an effect mediated by ET-1. ET-1 does not have a mitogenic effect on MCF-7 and MDA MB 231 breast cancer cell lines.

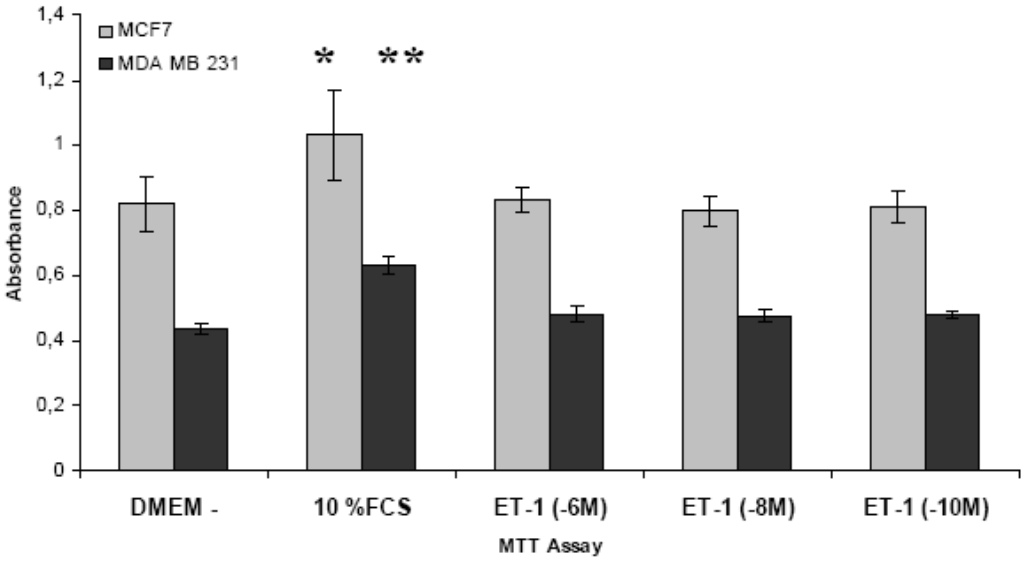


Figure 57: Analyses of cell viability by employing the MTT assay. Growth of MCF 7 cells were illustrated with light grey columns and MDA MB 231 cells were illustrated with dark grey columns. Cells were incubated 24 h with different concentrations of ET-1. 10% FCS containing medium was used as positive control for this viability test. (n=3, Student´s T-test, P<0.05; (*) MCF 7, (**) MDA MB 231).

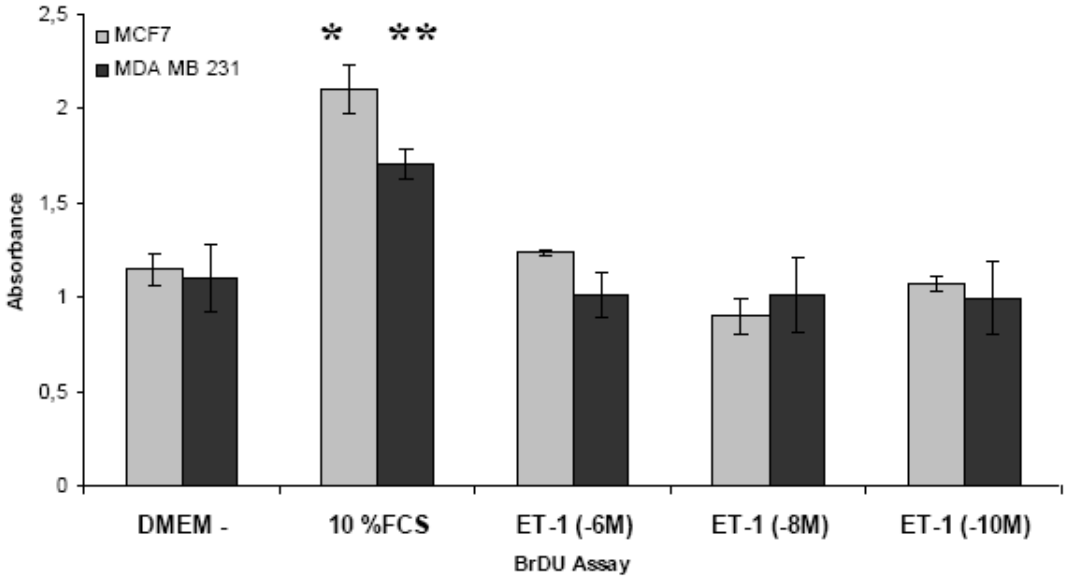


Figure 58: Analyses of cell viability by employing the BrDU assay. Growth of MCF 7 cells were illustrated with light grey columns and MDA MB 231 cells were illustrated with dark grey columns. Cells were incubated 24 h with different concentrations of ET-1. 10% FCS containing medium was used as positive control for this viability test. . (n=3, Student´s T-test, P<0.05; (*) MCF 7, (**) MDA MB 231)

# 4 Discussions

## 4.1 Identification of ET-1 expression in mammary glands

Like many peptide and growth factors, the presence of ET-1 in milk has suggested a physiological role for the lactating mother and the suckling neonate (Lam, et al., 1990). The gene expression of ET-1 has been reported particularly during the pregnancy and lactation period (Kozakai, et al., 2002). Moreover, the gene expression of ET-1 is strongly enhanced by prolactin (Baley, et al., 1990).

The present study was proposed to elucidate the possible functional roles of ET-1 in mammary gland development employing ET-1 transgenic mice. These animals express a genomic human ET-1 transgene comprising 8 kb of 5´ upstream sequences and 1 kb of 3´ sequences. Indeed, ET-1 transgenic mice have been extensively characterized for their cardiovascular and renal phenotypes (Hocher, et al., 1997). However, ET-1 transgene expression in the mammary gland and its influence on development and physiology has not been studied yet. Therefore, gene expression of ET-1 in the mammary glands was assessed by RT-PCR using special primers designed to either specifically amplify the human transgene or both, the human and the endogenous mouse transcript.

Study groups for the gene expression analysis of ET-1 are arranged by physiological and functional stages of the mammary gland. Therefore, 4 different developmental stages of the mammary gland were taken which were further divided into the 6 developmental phases; 8 weeks old virgin mice, 10th day of pregnancy (middle of pregnancy), $18^{th}$ day of pregnancy (late phase of pregnancy), $3^{rd}$ day of lactation (early phase of lactation), $14^{th}$ day of lactation (middle of lactation), $14^{th}$ day of involution (late phase of involution) (Figure 17)

According to PCR analyses, the expression pattern of the endogenous ET-1 gene differed from the ET-1 transgene. Whereas the mouse ET-1 gene was found to be expressed already in 8 weeks old adult virgin mice in both genetic groups (Figure 18B), the human transgene ET-1 was only detected during pregnancy and lactation periods (Figure 18 A). This discrepancy could be explained by the fact that the genomic ET-1 clone is still missing important regulatory sequences located either 5´or 3´, respectively, for mimicking the endogenous ET-1 expression pattern. Hence a developmental specific expression of the transgene was not achieved.

The human ET-1 gene expression was only detected in mammary glands of pregnant and lactating transgenic females (Figure 18A), demonstrating that expression of the transgene was restricted to these two developmental stages. The expression level of total ET-1 in the mammary glands of those groups of animals was assessed by real-time PCR. As shown in

Figure 19, total ET-1 expression was increased to about 6 fold in the mammary glands of transgenic mice compared to wild type mice mammary glands during pregnancy as well as lactation, demonstrating a prominent overexpression of ET-1 at these two developmental stages (Figure 19), and this provides the basis for analysing the biological consequences of this enhanced expression of such a developmentally important gene.

ET-1 exerts its effect by binding to two distinct GPCRs, the ETAR and ETBR. The expression level of both endothelin receptors was determined using real-time PCR and western blotting (Figure 20, 21) and no difference could be detected between wild type and transgenic animals, suggesting that the increase in expression of ET-1 is not influencing the expression of its receptors in this biological system.

## 4.2 Impaired lactational competence in ET-1 transgenic mothers

ET-1 transgenic mice exhibited a developmental phenotype defined as lactational incompetence. This defect is revealed when the ET-1 transgenic females gave birth to newborns when it was noted that some of the newborns died very soon after birth and many litters thrived poorly. A detailed analysis of the numbers of newborns, successfully raised mice and animals reaching adulthood was recorded and a significant increase in the proportion of pups that died shortly after birth was observed (Table 13). A possible explanation for this finding might be the fact that ET-1 a well known vasoconstrictor peptide, is also upregulated in the uterus during the parturition and able to increase myometrial smooth muscle tone (Uchide, et al., 2001), (Olgun, et al., 2008) thereby preventing a controlled delivery of the fetuses during the important phase of parturition. Hence an increased myometrial contraction in ET-1 transgenic mice during the parturition could be the reason for an increased death rate observed in ET-1 transgenic mothers (5,6 %) compared to (0.2%) in wild type mothers (Table 13).

Besides this increase in the number of pups that died shortly after birth, mortality during the lactation period was elevated as well. Macroscopically, an absence of milk in the stomach was found in several pubs, indicating a lactational deficit. To validate this observation, mortality and growth rate of the newborns were monitored. To sustain an equal nourishment of lactation each ET-1 transgenic mother (n=17) and wild type mother (n=10) was limited to nourish only 7 newborn. The entire litters (189 pups) were weighed at birth and during lactation (Figure 22). When assessing the weight gain of the offspring, it was noted that pups suckled by transgenic mice gained significantly less weight than those suckled by wild type females. However the initial body weight at birth did not differ between the two genetic groups. There might still be an effect of the genotype of the pups. The only way to address this would be an appropriate foster experiment which has not been performed. However, the genotype of the pups seems not to be a determining factor for the presented phenotype of

the mammary gland morphology and it appears more plausible that these alterations are responsible for the observed effects on weight gain of the pups.

Pups suckled by transgenic mothers displayed a significantly lower weight gain during the first 11 days of lactation. In addition an increased neonatal death was revealed during the first 11 days of lactation, and characterised by the fact that these newborns had no milk in their wombs. Especially, there was no difference in handling of mothers and suckling pups between the groups that could account for any difference in neonate survival. This first 11 days of lactation are recognized as the critical phase for weight gaining of the pups which is mostly depending on performance of the mother. Furthermore in addition to milk, the pups start to consume solid food after 12 days of age (Ramanathan, et al., 2007). Indeed, the weight gain slope of transgenic animals became comparable to the wild type counterpart after the lactation day 11 suggesting that the provision of solid food possibly sustained the weight gain of pups. However it has been reported that the increased demand for milk from growing pups forge ahead to milk production throughout lactation. For example, it has been observed that WAP depleted mice demonstrate a reduced weight gain of the pups in the second half of the lactation period. Measurements of weight gain or loss is important in assessing the nursing ability. Reduced weight gain and increased neonatal death are common phenomenon for a lactational defect which is mostly exhibited in genetically engineered mice (Palmer, et al., 2006).

### 4.2.1 Analysis of lactation defects in transgenic mice

Genetically modified animal models have been widely used to elucidate the functional aspects of the mammary gland development and lactation and the involvement of certain genes. Depending on their role and importance in the biology of mammary gland development, these experimental approaches resulted in lactational incompetence as a result of impaired alveolar differentiation, deficient synthesis or secretion of milk components, or deficits in milk letdown, respectively (Palmer, et al., 2006).

As listed in table 14, genetic modifications, such as overexpression or knock out of a specific gene were found to cause various types of defects. Histological and molecular differences in these animal models delineate the role of these specific genes which are involved in various cellular and molecular processes of mammary gland development and lactation. The histological analysis of the mouse mammary glands from the various models revealed cardinal features of lactational incompetence such as reduced ductal outgrowth, collapsed alveoli, increased adiposites, reduced alveolar expansion and persistence of CLDs during lactation. All these histological changes are exhibited as common features (stereotype) of the experimental models despite of their individual role of genes in mammary gland development and lactation.

In fact, lactational defects rarely occur naturally. Histological changes only take place when metabolic or secretory pathways are damaged due to altered regulation of a specific gene. Those damages can affect the expression of milk proteins, transport mechanisms, tight junction functionality, lipid metabolism or cellular fate which are related with the function of that specific gene. Therefore, in line with the obtained results from the ET-1 transgenic mouse model presented in this work, the effects of ET-1 transgene expression on lactational defects are compared at the cellular and molecular level with other animal models.

*Table 14: Phenotype of genetically engineered mouse models of lactation*.

| Gene | Modification | Phenotype | Reference |
|---|---|---|---|
| Prolactin | ko | Failure to lactate; defective mammary gland development | (Ormandy, et al., 1997) |
| STAT5 | ko | Failure to lactate; lobuloalveolar defects | (Liu, et al., 1997) |
| STAT5 | Transgene | Enhanced growth of pups delayed involution | (Iavnilovitch, et al., 2002) |
| Jak2 | ko | Failure to lactate; impaired alveologenesis | (Wagner, et al., 2004) |
| oxytocin | ko | Secretory defect, focal precocious involution | (Wagner, et al., 1997) |
| oxytocin | Transgene | Secretory defect | (Ho; Murphy, 1997) |
| EGFR | ko | Impaired maternal lactation | (Fowler, et al., 1995) |
| TGFβ-1 | Transgene | Failure to lactate; lobuloalveolar defects, reduced expression of milk proteins | (Jhappan, et al., 1993) |
| Amphiregulin | ko | Impaired lactation due to reduced ductal outgrowth and alveolar differentiation | (Luetteke, et al., 1999) |
| LIF | ko | Delayed involution | (Kritikou, et al., 2003) |
| c src | Transgene | Hyperplasia | (Webster, et al., 1995) |
| c src | ko | Failure to lactate; secretory defect | (Watkin, et al., 2008) |
| PKN-1 | Transgene | Secretory defect, precocious involution | (Fischer, et al., 2007) |
| WAP | ko | Pups fail to thrive during second half of lactation | (Triplett, et al., 2005) |
| WAP | Transgene | Impaired alveolar development | (Burdon, et al., 1991) |
| Beta-casein | ko | Reduced pup growth secretory defect | (Kumar, et al., 1994) |
| Alpha-lactalbumin | ko | Secretory defect, reduced pup growth | (Stinnakre, et al., 1994) |
| AKT | Transgene | Reduced pup growth, secretory defect | (Schwertfeger, et al., 2001) |
| HIF1 | Transgene | Reduced pup growth, secretory defect | (Seagroves, et al., 2003) |
| ProteinC | Transgene | Lactation failure of alveoli to expand | (Palmer, et al., 2003) |

## 4.3 ET-1 transgenic mice exhibited a secretory activation defect

ET-1 transgenic mice exhibited a normal pregnancy with well developed ductal branching and lobuloalveolar architecture. However these animals exhibited a lactational incompetence suggesting that ET-1 overexpression can affect the functionality of the mammary glands.

The primary function of the mammary gland is to supply a balanced mixture of nutrients for neonatal growth. This functional differentiation of the mammary gland is termed lactogenesis

and distinguished in two stages; secretory differentiation and secretory activation (Pang; Hartmann, 2007). Cellular differentiation for secretion begins at the middle of the pregnancy with an increased expression of milk protein genes. Characteristic features of the late phase of pregnancy indicating secretory differentiation are cytoplasmic lipid droplets (CLD) (Anderson, et al., 2007), (Richert, et al., 2000). These were found in both wild type and ET-1 transgenic mammary glands at the late stage of pregnancy. The distribution of the epithelial cells within the fat pad and density of the epithelial cells did not differ between both genetic groups (Figure 23, 24, 25, 26). Hence, differentiation of the mammary epithelium was not affected in transgenic animals at this developmental stage.

The secretory activation occurs after parturition and expression of milk protein genes (WAP, Beta-casein, and Alpha-lactalbumin) increases further (Rudolph, et al., 2007). Tight junctions, sealing the alveoli become impermeable and CLDs and casein micelles move to the alveolar lumen. With the suckling stimuli the first milk containing CLDs are secreted and disappear from the mammary gland in the first 2 days of lactation (McManaman; Neville, 2003).

At this time point, the histology of the mammary gland is generally characterised by the presence of fully expanded lumens surrounded by a densely stained layer of epithelial cells (Richert, et al., 2000). With the suckling stimuli stored milk is removed from the alveoli and alveolar epithelium expansion is synchronized by equally changes in other tissue compartments, while adipocytes lose their cellular contents (Oakes, et al., 2006). In wild-type mice, due to the pressure of accumulated milk in the alveoli a flattened appearance of the epithelium is observed. Although, ductal and alveolar structures developed normal during pregnancy (Figure 28, 31), the ET-1 transgenic glands displayed focal areas with collapsed alveoli and persistence of intracellular lipid droplets within the alveoli at the 3$^{rd}$ day of lactation. Trapping of these lipid droplets within the epithelial cells suggest a secretory activation defect. Therefore, nutrition of ET-1 transgenic pups was poor during the first half of lactation.

Removal of milk from alveoli is elicited by the suckling or milking stimulus. This process is accomplished by contraction of the myoepithelial cells surrounding the alveoli and ducts. Oxytocin which is released in response to the suckling stimulus from the mammary nipple causes the contraction of the myoepithelial cells surrounding alveoli and ducts.

ET-1 is known as a vasoactive substance that regulates the vascular tone by inducing contraction of myoepithelial cells (Yanagisawa, et al., 1988). This functional similarity between ET-1 and oxytocin suggests that the ET-1 may have a potential role on milk removal. Indeed, the presence of large CLDs post-partum was also noted in oxytocin transgenic bovine mammary glands exhibiting a similar phenotype as the ET-1 transgenic mammary glands (Anderson, et al., 2007), (Ho; Murphy, 1997). It was demonstrated that large doses of oxytocin treatment can lead to changes in the milk composition consistent with increased tight

junction permeability (Nguyen; Neville, 1998). Further increased tight junction permeability was associated with a decrease in milk secretion (Stelwagen, et al., 1997). Therefore, according to the phenotypical and functional similarities to oxytocin, a conjectural role of ET-1 on tight junction permeability was analysed.

During pregnancy, the tight junction complex is permeable for entry of the substrates into the alveolar lumen. Following the parturition the tight junction system mediates sealing of the alveolar epithelium to avoid milk leaking from the alveolar lumen to the basal area. (McManaman; Neville, 2003). The family of claudin proteins is involved in the regulation of the tight junction composition (Furuse, et al., 2002). As the claudin proteins are implicated in tight junction strand regulation (Furuse, et al., 2002) it was noted that claudin 8 expression increases as the mammary gland progresses from mid-pregnancy to functional lactation (Seagroves, et al., 2003). Further, a decreased claudin 8 expression was implicated in an increased tight junction permeability resulting in a secretory defect in the mammary gland (Seagroves, et al., 2003). Therefore, claudin 8 gene was specifically chosen here to analyse its expression on the RNA level.

As reported in results 3.4.2, no significant difference was obtained for claudin 8 gene expression in transgenic mammary glands compared to wild type counter part, neither during pregnancy nor during lactation. Therefore, ET-1 overexpression may not have an influence on claudin 8 expression and thereby also might not have an effect on the tight junction permeability which is in contrast to oxytocin. However, a secretory defect mediated by an increase of tight junction permeability has been demonstrated in other genetically modified animal models. Hence, other proteins involved in tight junction functionality such as other claudin family members might be involved in the mediation of the observed phenotype. Following this, future investigations should analyse if ET-1 overexpression can induce alterations in the structural components of tight junction architecture.

### 4.3.1 Differentiation of mammary gland cells and alveolar expansion in ET-1 transgenic mammary glands

The acquisition of the secretory capacity begins around the middle of the pregnancy with increased expression of milk protein genes (Naylor, et al., 2005). In mice, milk protein gene expression occurs in a programmed pattern such that Beta-casein is expressed during the middle of the pregnancy and later near the end of gestation WAP and Alpha-lactalbumin are expressed (Neville, et al., 2002).

It has been reported that WAP is a dispensable protease inhibitor to maintain the stability of secretory proteins in the milk (Triplett, et al., 2005) and in addition WAP was considered as a marker for terminal differentiation of the mammary secretory phenotype (Robinson, et al.,

1995). However, the expression of the WAP gene is not required for the functional differentiation of the alveoli (Triplett, et al., 2005).

In ET-1 transgenic mice WAP expression was found to be reduced to around 10% of its normal level (Results 3.4.1). Similarly to ET-1 transgenic mice, the mammary glands of WAP knockout mice exhibited well differentiated secretory acini during pregnancy. Following the parturition both animal model showed relatively smaller alveoli than their wild type counterparts (Triplett, et al., 2005). As discussed before, the first 11 days of lactation represent a critical phase for the weight gain of the pups which is mostly depending on the performance of the mother (Ramanathan, et al., 2007). Indeed, a reduced weight gain of suckling pups was observed during the first 11 days of lactation in the ET-1 transgenic model. Contrarily, pups were normally nourished during the first 10 days in WAP knockout mice. And these females exhibited a lactational incompetence, in particular during the second half of lactation. This observation suggests that WAP expression may be essential for the adequate nourishment of the growing young (Triplett, et al., 2005).

In ET-1 transgenic mice WAP is strongly downregulated in both, pregnancy and lactation periods, indicating that the overexpression of ET-1 may have an influence on the WAP gene expression. To address the question whether the reduction of WAP gene expression is a direct consequence of ET-1 overexpression or rather reflects an indirect effect, the employment of an in vitro experimental model would be preferential. However, there is no potential cell culture model available that could mimic lactational switches in vitro. In fact, it has been extensively proved that the WAP gene is specifically expressed in mammary tissues and is maximally induced by lactogenic hormones at mid pregnancy and lactation (Hobbs; Rosen, 1982), (Liu, et al., 1997). Breast cancer cells are of mammary origin but they do not express the WAP gene (Lubon; Hennighausen, 1987). Therefore, the WAP gene expression analysis was omitted from the in vitro studies.

In addition to the downregulation of WAP, ET-1 transgenic mice exhibited also downregulation of Beta-casein expression during the lactation period. Caseins play an important role in cell uptake of physiologically important ions such as calcium and phosphate through formation of soluble ingredients (Sokolovski, et al., 2008). Further Beta-casein knockout animals demonstrated a secretory defect with reduced weight gain of suckling pups (Kumar, et al., 1994). It has been reported that WAP has no effect on Beta-casein and Alpha-lactalbumin mRNA expression (Triplett, et al., 2005). Therefore the reduced WAP and Beta-casein expression together induced the development of lactational incompetence with a stronger phenotype in ET-1 transgenic mice than in WAP knockout mice.

It has been noted that STAT5 transgenic mice showed elevated levels of caseins with increased weight gain of suckling pups (Iavnilovitch, et al., 2002). STAT5 is known as a critical

regulator for transcriptional activation of the milk protein genes such as WAP and Beta-casein (Liu, et al., 1997), (Iavnilovitch, et al., 2002). Activation of the STAT5 pathway by prolactin, GH, EGF and amphiregulin (Boutinaud; Jammes, 2004), (Miyoshi, et al., 2001), (David, et al., 1996) transduces signals to the secretory cells for functional differentiation and alveolar expansion. Activity of STAT5 is increased after parturition and throughout lactation (Liu, et al., 1997). Pointing to the importance of STAT5 activity, STAT5 depleted mice fail to lactate and exhibited disorganized epithelial architecture (Liu, et al., 1997), (Miyoshi, et al., 2001). In total, initiation of the alveolar expansion is controlled by the STAT5 pathway (Jaroenporn, et al., 2009).

Similarly, the ET-1 transgenic mice exhibited a decreased alveolar expansion as a result of the defect in secretory activation. Therefore, activation of STAT5 was analysed by measuring the relative phosphorylation level of STAT5 in both late pregnancy and early lactation. However, no significant differences were observed in the phosphorylation level of transgenic mammary glands compared to wild type mammary glands implicating that the ET-1 transgene has no influence on the activation of STAT5.

The STAT5 activity has been considered as a marker for secretory differentiation and alveolar expansion. As defined before, the milk proteins were regulated in response to STAT5 activity. However, in ET-1 transgenic mice STAT5 activity did not differ compared to wild type at lactation day 3, but WAP and Beta-casein gene expressions were found to be down regulated. Based on these findings it can be concluded that the overexpression of ET-1 can influence the WAP gene expression independently from STAT5 activity. Indeed, it has been reported that the regulation of WAP gene expression is determined by the cooperative interaction of a number of transcription factors with several binding sites in the promoter which is arbitrarily divided in two DNA binding regions (Ozturk-Winder, et al., 2002).

In line with the obtained results the reduced weight gain of suckling pups in the first half of lactation could be related with a downregulation of Beta-casein but not WAP expression. Indeed it was noted that removal of the pups resulted in a rapid downregulation of Beta-casein levels in the mammary cells (Iavnilovitch, et al., 2002), (Kumar, et al., 1994). While STAT5 activity is normal, a fall of Beta-casein expression suggests that some other parameters could also affect the Beta-casein expression in addition to STAT5 activity. However, gene expression of Beta-casein was not effected by the ET-1 transgene expression during pregnancy. This suggests that the downregulation of Beta-casein during lactation should be a consequence of the lactational defect but not vice versa.

### 4.3.2 Regulation of the synthesis of other nutritional compounds in ET-1 transgenic mammary glands

Lactation is defined as copious production of milk to support the nutritional needs of the suckling pups. The milk contains 12% proteins (WAP, caseins, Alpha-lactalbumin etc), 30% lipid and 5% lactose (Anderson, et al., 2007). Lactose, a disaccharide that is unique to milk is not detected in the mouse mammary gland until the day before parturition.

With the onset of lactation the transfer of sugars from the blood to the milk is blocked by the closure of the tight junctions. During lactation the secretory cells produce the lactose by a unique synthesis process. Therefore, the factors involved in lactose synthesis could be considered as markers for secretory activation. Prior studies indicated that the regulation of lactose and lipid synthesis in the mammary gland occurs at the level of mRNA expression (Rudolph, et al., 2007). Therefore, expression of some of the key genes was analyzed on the mRNA level. A large proportion of the fatty acid as well as lactose is synthesized from glucose. It was shown that the glucose level in the alveoli increased with the onset of secretory activation and decreased gradual with onset of weaning (Neville, et al., 1990). The process of glucose uptake is mediated by the glucose transporters in mammary gland.

GLUT1 is the major and the most extensively studied glucose transporter in the basal membrane of the alveoli (Anderson, et al., 2007). Its expression increases throughout late pregnancy and peaks during lactation, upon demand for lactose or fatty acid synthesis (Nemeth, et al., 2000). Consequently, GLUT-1 expression was analyzed in ET-1 transgenic mammary glands compared to wild type. However no significant difference was obtained in the expression level of GLUT-1 between the two genetic groups.

Another critical factor for lactose synthesis is the well known milk protein Alpha- lactalbumin which in combination with galactosyl transferase acts as lactose syntase in the synthesis of lactose in the mammary gland. Thereby, Alpha-lactalbumin represents the essential cofactor and is the limiting factor for lactose synthesis (Rudolph, et al., 2007). The very low expression of Alpha-lactalbumin to prevent inappropriate lactose synthesis during pregnancy is increased after the parturition and throughout the lactation period (Rudolph, et al., 2007). Alpha-lactalbumin knockout mice were found to be unable to nourish their pups and this defect is mediated by milk stasis due to the inability of the gland to eject highly viscous and fatty milk (Stinnakre, et al., 1994).

Gene expression of Alpha-lactalbumin is not altered in ET-1 transgenic mice, neither during pregnancy nor during the lactation period. In total, the real-time PCR analyses showed that the overexpression of ET-1 had no influence on the expression level of the major glucose transporter GLUT-1 and on Alpha-lactalbumin, a critical factor in lactose synthesis (see 3.4.2).

Milk contains around 30% lipids which are derived from a glycerol backbone and esterified fatty acids. These lipid precursors are supplied either with the diet, from adipose tissue stores, or synthesized *de novo* in the mammary gland. Further, desaturation and elongation of fatty acids are processed predominantly in the mammary gland (Anderson, et al., 2007). To support the synthesis of milk lipids as well as lactose there must be a significant programming in the physiology of alveolar epithelial cells to direct metabolic precursors to the synthesis of these two compounds.

Regarding the secretory activation defect in the ET-1 transgenic mice, this represents another model of lactational deficiency (Table 14) by exhibiting a persistence of CLDs in the alveoli during lactation. Therefore, lipid biosynthesis in mammary glands could be recognized as another limiting factor for milk production (Anderson, et al., 2007) It was noted that the SREBF family of transcription factors are critical regulators of fatty acid and cholesterol biosynthesis (Anderson, et al., 2007). Consequently, regulation of lipid synthesis occurs at the level of mRNA expression (Rudolph, et al., 2007) and therefore SREBF-1 gene expression was analyzed to monitor the levels of lipid biosynthesis in the mammary glands.

In ET-1 transgenic mice SREBF-1 gene expression was found to be upregulated about 9 times compared to wild type mice at lactation day 3 (see 3.4.2). This result could suggest that highly viscous and fatty milk is causing the observed histological alteration in ET-1 transgenic mammary glands. However, SREBF-1 gene expression was found to be normal at pregnancy day 18, indicating that overexpression of ET-1 has no direct influence on SREBF-1 expression at this developmental stage. Nevertheless, as pointed out SREBF-1 was significantly upregulated during lactation reflecting the histological alterations of secretory defect with persistence of CLDs. It has been noted that regulation of SREBF-1 is controlled by AKT (Schwertfeger, et al., 2001). Similar to ET-1 transgenic mice, constitutively active AKT transgenic mice exhibited a secretory activation defect with persistence of CLDs in alveoli and upregulation of SREBF-1 expression (Anderson, et al., 2007), (Schwertfeger, et al., 2001).

AKT1 is a unique member of the AKT family that is upregulated in the mammary gland during pregnancy and lactation (Creamer, et al., 2001). AKT plays a central role in regulating glucose transport, glucose uptake and GLUT-1 expression for milk producing cells (Barthel, et al., 1999). Further, AKT explicitly controls the lipid metabolism by regulating SREBF-1 gene expression in the mammary gland during lactation (Schwertfeger, et al., 2001). Therefore AKT activation was analysed in ET-1 transgenic mice during both periods, pregnancy and lactation. Activation of AKT was monitored by the analysis of its phosphorylation status. However no differences were found in the activation of AKT between ET-1 transgenic and wild type mammary glands at both developmental stages.

In line with the obtained results, ET-1 overexpression had no influence on AKT function and GLUT-1 expression which is under the control of AKT. In addition, as described before SREBF-1 was found to be increased about 9 times at lactation day 3. In fact, SREBF-1 is thought to be stabilized at secretory activation by AKT (Schwertfeger, et al., 2001). Probably this contrariety at lactation day 3 is derived from samples which were obtained from homogenized ET-1 mammary glands containing large amounts of adipocytes which were found in histology and thereby influencing the SREBF-1 expression level. Taken together, the cause of an increased SREBF-1 expression might be artificially influenced in the ET-1 transgenic mice.

In fact, an increase of adipocytes in mammary glands during the lactation period indicates precocious involution. It is known that AKT plays a central role in promoting survival of the mammary epithelial cells during lactation. Therefore AKT expression is high during lactation. With the onset of involution AKT expression and activation is decreased (Schwertfeger, et al., 2001). The ET-1 transgenic mice displayed a downregulation of AKT expression on the protein level at lactation day 3 but not at pregnancy day 18, suggesting that the initiation of precocious involution could be the reason for the downregulation of AKT.

### 4.3.3 ET-1 transgenic mice exhibited precocious involution

Involution is defined as a transition of the mammary gland from the lactating stage to the non-lactating stage in preparation for a subsequent pregnancy. However, already at an early stage of lactation, ET-1 transgenic mammary glands histologically exhibited focal precocious involution. Involution of the mammary gland is a two steps process because of its reversibility (Lund, et al., 2000). The first phase is reversible and characterised by the accumulation of local factors. At this stage pro-apoptotic factors are upregulated while survival factors are reduced. Through the re-suckling stimuli, the involution process is reversed. In contrast, the next phase is not reversible and widespread apoptosis and tissue remodelling takes place (Stein, et al., 2007). Several possible mechanisms can trigger involution. The involution elicits after cessation of milking and sudden weaning of the offspring or gradual decrease in suckling. Consequently, absence of suckling stimuli or milk stasis (Green; Streuli, 2004), (Watson, 2006) results in rapid alteration of gene expression which modulates key proteins involved in the involution process of the mammary gland (Sutherland, et al., 2007). These molecular changes in the epithelial cells of the mammary gland lead to activation of STAT3 which is critical for the initiation of involution (Chapman, et al., 1999), (Kritikou, et al., 2003). In response to STAT3 activation the nucleus and cytoplasm condense, the chromatin becomes fragmented and apoptotic cells are scattered into the alveolar lumen (Strange, et al., 1992).

Remarkably, the ductal tree formation of lactating ET-1 transgenic mammary glands was similar to that of a pregnant transgenic gland, although some alveolar structures persist in whole mount slides (see 3.3.3). Further, histology of these sections displayed focal collapsed alveoli into clusters of epithelial cells and increased amount of adipocytes that appeared to be refilling the rest of the area. These histological alterations points to a process of precocious involution taken place in the mammary glands of ET-1 transgenic mice. Consequently, the activation of STAT3 was analyzed at pregnancy day 18 and at lactation day 3. At pregnancy day 18 STAT3 activity was not detected in both groups (see3.5.1). However, the relative phosphorylation level of STAT3 was found to be significantly increased at lactation day 3, demonstrating that this critical factor for the initiation of involution exhibited a higher activity in the ET-1 transgenic mammary glands compared to wild type mammary glands.

As a consequence of the defective secretion, the ET-1 transgenic mammary glands are characterised by precocious involution which is triggered by the STAT3 activation. STAT3 is activated in response to a number of cytokines including members of the cytokine family such as IL-6 and LIF (Zhao, et al., 2004), (Akira, et al., 1994). Consequently, the expression of IL-6 and LIF genes was assessed using quantitative real time PCR. LIF expression was found to be significantly upregulated in ET-1 transgenic mice whereas IL-6 expression remains unchanged at the 3$^{rd}$ day of lactation (see 3.5.2).

LIF is known as the principal physiological activator factor for both, STAT3 activation and the subsequent involution process in the mammary gland (Kritikou, et al., 2003), (Zhao, et al., 2004). LIF is expressed at low but detectable levels in post pubertal, adult virgin, and pregnant mouse mammary glands. Following the parturition, the LIF expression drops and become almost undetectable in lactating glands. Shortly after weaning LIF expression shows a steep increase which is maintained for the following 3 days (Schere-Levy, et al., 2003) and thereby inducing involution of the mammary gland. It was noted that milk stasis induces the expression of LIF resulting in precocious involution (Green; Streuli, 2004), (Watson, 2006). Hence, it comes not as a surprise to detect increased LIF expression in ET-1 transgenic mammary gland.

Interestingly, LIF expression was not only upregulated at lactation day 3 but also at pregnancy day 18 in ET-1 transgenic mammary glands (see 3.5.2). This finding implies that overexpression of ET-1 has an influence on upregulation of LIF expression during both developmental stages, which might render the mammary gland to an even more pronounced phenotype. This finding has led to the further in vitro analyses of the ET-1 induced regulation of LIF which could also provide some insights into the regulation of these molecular processes. Therefore, two cell culture models, MCF7 cells and MDA MB 231 cells, respectively, were treated with 10mM active ET-1 for 10 min. However, no significant differences were obtained for the activation of LIF expression when compared to non treated cells (see 3.5.2).

However, limitations of the in vitro models, e.g. both cell lines are of adenocarcinoma origin, may be responsible for these negative results used to demonstrate this interaction. Therefore the type of cells and their origin determines their physiological responses. As displayed before LIF gene expression was upregulated in both pregnancy and lactation periods. Interestingly, the increased levels of LIF expression did not effect the STAT3 phosphorylation at pregnancy day 18. Apparently, LIF alone is not sufficient to activate STAT3 during pregnancy. This finding suggests that LIF functions different in lactation and pregnancy in virtue of different physiological conditions and hormonal regulations. It has been noted that LIF mediates its function in reciprocal succession through ERK1/2 activation; therefore STAT3 activation is initiated by distinct pathways in different development stages of the normal mammary gland (Kritikou, et al., 2003). For example, LIF induces mammary gland involution during lactation (Kritikou, et al., 2003) while LIF mediates branching morphogenesis during early mammary development (Zhao, et al., 2004) and induces cellular proliferation in breast carcinomas (Levy, 1997). Probably this functional switch of LIF could prevent the inappropriate induction of apoptosis during pregnancy.

As described, ET-1 transgenic mice share similar histological patterns with other transgenic models due to a defect on secretory activation. Despite suckling and the presence of systemic lactogenic hormones, ET-1 transgenic mice exhibited focal precocious involution at the 3$^{rd}$ day of lactation.

Mothers continued to nourish their pups throughout the lactation period. Meanwhile, the involution process continues slowly and affects either parts of the mammary gland or the entire mammy gland. As already mentioned the reversible phase of involution can be suppressed or elongated by various survival signal pathways like AKT and STAT5 (Schwertfeger, et al., 2001), (Fata, et al., 2001). Indeed, ET-1 transgenic mice demonstrated a normal STAT5 and AKT activity during lactation. Possibly these survival factors could sustain the delay of involution through the lactation period. In fact, constitutively activated AKT transgenic mice demonstrated a delay in post-lactational involution (Schwertfeger, et al., 2001). However this transgenic model exhibited a lactation defect accompanying persistence of CLDs in alveoli which results in a 50% decrease in litter weight over the first 9 days of lactation, although ductal structures and alveolar units developed normally during pregnancy (Schwertfeger, et al., 2001).

With that respect initiation of precocious involution is caused by a defective secretion which was described in stereotypical alterations in genetically modified animals. However, the causative effect of the ET-1 transgene on LIF gene expression and functional consequence of this interaction should be further analyzed.

## 4.4 ET-1 transgenic mice developed lactational hyperplasia

ET-1 transgenic mice represent impaired lactational incompetence by virtue of a secretory activation defect. At the middle of lactation, ET-1 transgenic mammary glands showed an increase in adipose tissue, in which collapsed secretory alveoli were found (see 3.3.3 Figure 28). In fact, these histological changes were reminiscent of the morphology seen during involution of the mammary gland (Richert, et al., 2000).

Besides the delineated similarities in the molecular and histological characteristics between the ET-1 transgenic females and the other genetically modified mouse models outlined in Table 14, all ET-1 transgenic mammary glands displayed focal and intense lobular proliferation in various grades which was not found in wild type mammary glands. This unusual histological formation was characterised by markedly enlarged lobules with increased cytoplasm volume and enlarged nuclei. Further, alveolar organization was lost in parts of these areas, which is referred to an abnormality of development and indicative of an early neoplastic process. However, no definite signs of dysplasia were found (see 3.3.4, Figure 31).

It is known that neoplastic progression is characterized by the loss of normal tissue architecture, including polarity. Generally primary breast carcinomas show a dramatic increase in the ratio of luminal to myoepithelial cells, and many invasive breast carcinomas essentially lack myoepithelial cells entirely (Gusterson, et al., 2005).

An immunocytochemical method for the detection of myoepithelial and epithelial cells was employed to distinguish the normal mammary from the neoplastic mammary gland (Gusterson, et al., 1982), (Gusterson, et al., 2005). Indeed, routine histopathological evaluations of mammary glands use the retention of the myoepithelial layer as a critical diagnostic criterion to distinguish from mammary carcinoma (Jones, et al., 2004). Therefore, immunohistostaining of smooth muscle actin (SMA) was employed in order to rule out an underlying malignant transformation. SMA immunostaining in ET-1 transgenic mammary glands clearly manifests the non neoplastic structure with the presence of myoepithelial cells surrounding alveoli (figure 32). Taken together, these findings are indicative of lactational hyperplasia.

Lactational hyperplasia is known to be a benign breast lesion that regresses spontaneously after lactation and no malignant potential has been ascribed to this tissue lesion (Saglam; Can, 2005). The regression of lactational hyperplasia is maintained by apoptosis (Sabate, et al., 2007). Indeed, lactational hyperplasia was not detected during involution in ET-1 transgenic mammary glands (see 3.3.4, Figure 33).

Moreover, detailed histological analyses of mammary glands from ET-1 transgenic females which had undergone several rounds of pregnancy and lactation were performed in order to analyse if the multiple inductions of the ET-1 transgene expression during these develop-

mental stages can induce any neoplastic transformation in the mammary epithelial cells. However, due to the lactational defects observed in the mammary glands of the transgenic females and the increase of these abnormalities with the increasing number of pregnancies this analysis could not be performed successfully (data not shown).

It was noted that lactational hyperplasia is characteristically thought to have undergone certain histological changes owing to the physiologic state (Sabate, et al., 2007). However, this study demonstrated that all ET-1 transgenic mice are prone to develop lactational hyperplasia in various sizes at lactation day 14.

ET-1 has also been associated with a wide range of biological activities including mitogenic and proliferative responses in vascular smooth muscle cells and other cell types (Bagnato, et al., 1997). Besides its contribution to developmental processes (Kurihara, et al., 1994) augmented ET-1 expression has been demonstrated in a variety of solid tumours thereby promoting growth and inhibiting apoptosis in breast cancer while decreasing tumour cell differentiation (Alanen, et al., 2000); (Bagnato, et al., 2001). It has been noted that some cancer cells secrete ET-1 in vitro and that ET-1 also promotes tumour cell growth (Pfab, et al., 2004). Therefore in order to evaluate the effect of ET-1 on breast cancer cells in vitro, the cells were stimulated with ET-1. However, ET-1 treatment did not exert any proliferative effect on the two breast tumour cell lines in vitro. Meanwhile, another identical study has been published by Hagemann demonstrating similar results about the effect of ET-1 treatment on cellular behaviour of breast cancer cell lines. Additionally, this study represented that the level of ET-1 expression by tumour cells correlates with their invasiveness (Hagemann, et al., 2005). However, a causative effect of upregulated ET-1 gene expression on the induction of proliferative lesions such as lactational hyperplasia has not been reported in vivo.

ET-1 exerts its effect by binding to two distinct G Protein Coupled Receptors (GPCR), the Endothelin A (ETAR) and B Receptor (ETBR). Although, both receptors are highly homologous on the DNA level, they exhibit functional differences in tissue distribution and coupling to G proteins (Levin, 1995). For example, the ETAR can stimulate cAMP through Gs, whereas the ETBR inhibits forskolin-induced cAMP by interaction with Gi (Takagi, et al., 1995). Furthermore, ETAR predominantly mediates vasoconstriction in vascular smooth muscle cells (Marsault, et al., 1993) whereas, ETBR activation results in vasodilatation in vascular endothelial cells (Yanagisawa; Masaki, 1989). Hence, endothelin receptors are able to stimulate multiple signaling systems by differential coupling to G proteins. However it remains to be established which of the two endothelin receptors, and how these receptors are involved in inducing the mammary gland phenotype in the ET-1 transgenic mice.

Endothelin receptors exert their effect by the recruitment of second messenger systems leading to the subsequent phosphorylation of the target proteins including ERK and AKT activa-

tion, (Kasuya, et al., 1994), (Taurin, et al., 2007) which are responsible for the activation of mitogenic and survival signals (Rozengurt, 2007). In response to activated GPCR pathways these mitogenic signaling activities are also strongly implicated in tumour processes (Rozengurt, 2007).

Indeed, ET-1 transgenic mice were found to exhibit a significant increase in ERK1/2 activities in both, pregnancy and lactation periods. An increased ERK1 activity was found to suppress apoptosis (Iwasawa, et al., 2009). Hence, this enhanced ERK1 activation could be a possible factor for an extension of the first reversible phase of involution which was demonstrated to be initiated during early lactation. In addition, changes in the regulation of ERK activity can guide the cells to undergo tumourigenic transformation as was demonstrated in renal cell carcinoma (Oka, et al., 1995) and breast cancer (Sivaraman, et al., 1997)

Endothelin receptors are also able to communicate with structurally unrelated receptors such as the EGFR (Cazaubon, et al., 1994) resulting in EGFR characteristic intracellular signals. The EGFR mediated signaling pathway is positioned to affect duct formation as well as the outgrowth and branching of the mammary gland during pregnancy (Wiesen, et al., 1999), (Hynes; Watson, 2010). Daub and co-workers recognized the engagement of GPCRs and EGFR as an essential signaling network for mitogenesis (Daub, et al., 1996). Further EGFR transactivation or receptor crosstalk was found to be important during morphogenesis and organogenesis. Indeed, recent studies reported that morphogenesis of the mammary gland requires paracrine activation of the EGFR via metalloprotease dependent shedding of amphiregulin (Zhang, et al., 2006), (Ciarloni, et al., 2007).

Transactivation of the EGFR is regulated by various cellular responses such as overexpression, amplification or mutation of critical pathway elements with variable functional outcomes which are frequently linked to hyperproliferative diseases. For example GPCR induced EGFR transactivation was found to mediate cell proliferation in breast cancer cells (Greco, et al., 2003), (Muscella, et al., 2003) and an increase of tumourigenicity in ovarian cancer cells (Rosano, et al., 2007).

Therefore, a growing interest in the analysis of the receptor crosstalk and functional consequences of EGFR activation on proliferative diseases evoke to the analyses of in vivo aspects of EGFR transactivation. These aforementioned findings could be related with the development of lactational hyperplasia seen in the ET-1 transgenic mammary glands.

As mentioned before, EGFR activation is crucial for ductal branching during the pregnancy (Fowler, et al., 1995), (Xie, et al., 1997). Further, immunoblot analysis had demonstrated high levels of EGFR during puberty, pregnancy and involution as well as during sexual maturity, and low levels throughout lactation (Darcy, et al., 1999). Indeed, initial western blot studies performed with mammary glands derived from ET-1 transgenic mice were not able to

detect an EGFR signal during the lactation period. Therefore immunoprecipitation was employed to detect total EGFR and phospho EGFR. Structurally, EGFR contains multi phosphorylation sites in intracellular domains, and several tyrosine (Y) phosphorylation domains (Y845, Y998 and Y1068) were analyzed during initial studies on EGFR activation (data not shown). In addition, it has been reported that various GPCR agonists including ET-1 could stimulate the phosphorylation of Y845 in the EGFR in vitro (Boerner, et al., 2005).

Finally, densitometric analyses revealed that the Y845 residue of the EGFR was more phosphorylated in ET-1 transgenic mammary glands in comparison to wild type mammary glands at lactation day 14 (see results 3.6.1). Activation of EGFR affects a wide range of cellular responses, depending on the coordinate expression of the cognate ligand (Peles; Yarden, 1993). Up to now, eight EGF like ligands that directly activate the EGFR have been identified: EGF, TGFα, heparin binding-EGF (HB-EGF), amphiregulin, betacellulin, epiregulin, epigen and cripto. (Cohen, 1986), (Luetteke; Lee, 1990), (Higashiyama, et al., 1992), (Shoyab, et al., 1988), (Riese, et al., 1998), (Strachan, et al., 2001), (Salomon, et al., 1999). Each of these molecules activates receptors of the HER family of tyrosine kinase receptor by autocrine or paracrine stimulation. Further, upregulation of these ligands is believed to be important for tumour growth (Troyer; Lee, 2001). Therefore, the most widely expressed ligands TGFa, EGF, HB-EGF and amphiregulin, involved in the activation of the EGFR, were analyzed using real-time PCR. As presented in the result section (results 3.6.2), amphiregulin expression was significantly upregulated in ET-1 transgenic mice at lactation day 14. None of the three other ligands did exhibit significant differences in their expression level.

It was demonstrated that amphiregulin is a unique EGFR ligand for ductal branching and ductal outgrowth (Jackson, et al., 2003), (Luetteke, et al., 1999). Moreover, knockout of EGF, TGFa, HB-EGF and betacellulin alone or in various combinations neither affect ductal outgrowth nor lactation (Luetteke, et al., 1999). Due to its function on ductal development of the mammary gland, amphiregulin expression is increased during pregnancy and decreased dramatically after parturition (D'Cruz, et al., 2002), (Schroeder; Lee, 1998). Amphiregulin gene expression is upregulated in ET-1 transgenic mice both during pregnancy and during lactation periods (results 3.6.2) which is suggesting that the regulation of amphiregulin is influenced by the ET-1 overexpression. Nevertheless, this finding was not supported by in vitro studies. Considering the complexity of the functional and developmental stages of the mammary gland and its hormonal regulation, there is not a single in vitro model which can mimic the structural and physiological function of the mammary gland such as pregnancy, lactation and involution. Therefore any causative effect of ET-1 expression on amphiregulin gene expression should be further investigated in ET-1 transgenic mice.

Amphiregulin expression is not only critical for mammary gland development but also strongly related with breast carcinomas and neoplastic progression (Sternlicht; Sunnarborg, 2008), (McBryan, et al., 2008). Amphiregulin appears to be the most abundant EGFR agonist which is breast cancer associated and expressed by these cells (Gilmore, et al., 2008). Several studies reported an increased amphiregulin expression in parallel to breast cancer. Therefore, amphiregulin was chosen as an pharmacological target for breast cancer treatment (Normanno, et al., 1995), (Willmarth; Ethier, 2008). As already mentioned an ET-1 upregulation was found in a variety of tumours (Alanen, et al., 2000), (Bagnato, et al., 2001), (Hagemann, et al., 2005). Hence, the ET-1 axis becomes an interesting new therapeutic target for advanced tumour diseases in clinical trials (Herrmann, et al., 2006). However, a causative effect of increased ET-1 expression on tumour induction and development is still not clear. In this study, the ET-1 transgenic mice exhibited a type of benign tumour defined as lactational hyperplasia during the middle of lactation. In order to understand the mechanism of hyperplastic progression the EGFR activity was analysed. It was reported that ET-1 can activate tyrosine kinase receptors such as EGFR by receptor transactivation (Daub, et al., 1996); (Boerner, et al., 2005). The mechanism of ET-1 induced EGFR transactivation might provide a molecular explanation how overexpression of ET-1 could regulate the proliferative behaviour of tumour cells. Indeed, ET-1 transgenic mice were found with an increased EGFR activation in their mammary glands during lactation. Moreover, EGFR activation is maintained by an increased amphiregulin expression (Gschwind, et al., 2003) which again might be regulated by the observed ET-1 overexpression. However no neoplastic transformation was detected in the mammary glands of the ET-1 transgenic females.

This study therefore highlights again the multiple roles of ET-1 which originally was found as a potent vasoconstrictive peptide and then was identified to contribute significantly to various other important developmental processes. As demonstrated this small peptide contributes prominently to the function of the mammary gland during pregnancy and lactation.

## 4.5 Perspectives

The involvement of the endothelin axis in tumourigenicity has been described in several reports (Alanen, et al., 2000), (Bagnato, et al., 2001), (Hagemann, et al., 2005). However none of these reports described the causative effect of ET-1 on inducing hyperproliferative lesions. In this study, ET-1 transgenic mice demonstrated a type of benign hyperplasia in the mammary gland. However, insights into underlying molecular mechanisms, direct experimental proofs, as well as validation are needed to clarify whether an ET-1 overexpression can contribute to mammary tumourigenicity.

Giving the significance of ET-1 in essential physiological functions and its potential role in inducing pathophysiological abnormalities in the mammary gland, future studies will have to

focus on mechanistic details of two ET-1 receptor signaling targets using therapeutic antagonists in vivo. Within this context molecular entities of the ET-1 axis in lactation pathophysiology could discerns coordinately regulated gene expression pathways and provides information about comparative histological changes. In addition to animal studies, a whole mount mammary gland organ culture method (Brandt, et al., 2000) can be considered for the analysis of the endothelin axis. This experimental model will ease to understand on a cellular and molecular level the contribution of various treatment regimens.

In order to address the transformation potency of ET-1 from a benign character to cell invasive character, cloning and transfection of the ET-1 gene into the non-cancerous breast cell lines (MCF10A, MCF12A) will be a useful strategy to identify signal specificity and signaling elements that link both endothelin receptors to these molecular processes.

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